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The Secretary of Health and Human Services has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1983.

Proceedings of the International Symposium on Methotrexate

Chairman: K. C. Calman

Coordinator: Colin P. Turnbull

Editors: A. Hilary Calvert
Colin P. Turnbull

November 1979
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Introduction to Proceedings

A. Hilary Calvert [1]* and Colin P. Turnbull [2]

Readers of these proceedings could be forgiven for questioning the need for a further symposium on methotrexate (MTX) which has, after all, been in clinical use for over 25 years. During the last 3 decades, however, revolutionary changes have occurred in the practice of clinical oncology that not only have concerned the utilization of individual drugs, but also have vastly widened the applications of cytotoxic chemotherapy in the treatment of human cancer. These latter changes have, at least in part, derived from the belated acceptance of the systemic nature of many common human malignancies at the time of their diagnosis. The resulting increase in the use of cytotoxic drugs has been accompanied by an increase in the expertise with which they are applied, which has been paralleled by a gain in the understanding of their optimal administration. In particular, intermittent schedules have gradually replaced continuous administration of the drugs and doses of drugs have been modified to suit individual patients. These innovations have perhaps had a greater effect on the clinical use of MTX than on that of any other drug. Such techniques as intermittent administration, the use of folinic acid (FA) rescue (1) and the monitoring of plasma levels have changed both the toxic manifestations (2) and the clinical spectrum of the drug (3) to such extent that one might speculate that it would scarcely be recognizable to Sidney Farber, who first introduced antifolate therapy. Thus, changes in the indications for cytotoxic chemotherapy in general, as well as refinements in the utilization of MTX in particular, fully justify the continuing evaluation of the potential contribution of the drug to cancer treatment.

As a cytotoxic agent MTX has a number of unique features. Unlike many agents it does not react chemically with nucleic acids. It is the only antimetabolite in common use that is not incorporated into nucleic acids, its action being wholly attributable to inhibition of the synthesis of essential DNA components (4). This unique biochemical property is probably associated with the fact that its cytotoxic effects may be abrogated by an antidote (FA) (5); furthermore, there are no documented carcinogenic effects of MTX (6,7), unlike many other cytotoxic agents that react chemically with or are incorporated into DNA (8,9). As a result of these properties, myelosuppression and gastrointestinal toxicity are wholly preventable by the appropriate use of FA. In addition, the chronic sequelae of many cytotoxic drugs (carcinogenesis and sterility) appear to be absent with MTX (6,10).

MTX has been shown to have a curative role in choriocarcinoma (11). It is also a component of the maintenance schedules used for the potentially curative treatment of childhood lymphatic leukemia (12). However, protocols used with curative intent for other tumors such as Hodgkin's and non-Hodgkin's lymphoma (NHL), testicular tumors, and certain childhood tumors rely heavily on steroids, alkylating agents, and vinca alkaloids (13–15). Although certain of these protocols have resulted in significant proportions of patients surviving long enough to justify the assumption that they may be cured of their original disease, this success has been somewhat tempered by the emergence of long-term side effects. Most notable of these are the high incidences of second tumors (8) and infertility (16). In the case of Hodgkin's disease, it has been suggested that second tumors are especially common when combined modality treatment comprising chemotherapy and radiotherapy has been used. With these observations in mind and in view of the lack of carcinogenic effects of MTX, a reappraisal of the drug in some of these diseases is perhaps warranted. Such considerations are also highly pertinent to the design of chemotherapeutic regimens for surgical adjuvant treatment where a substantial number of patients so treated are likely to survive for many years and are therefore at risk of long-term sequelae from chemotherapy.

Combination chemotherapy is currently widely used for many solid tumors, particularly breast cancer (17), head and neck cancer (18), testicular teratoma (15), and lung cancer (19), and has in many instances been shown to be superior to single-agent treatment in terms of both response rates and survival. Activity of a drug given as a single agent is regarded as a prerequisite for its inclusion in a combination. As a single agent, MTX is active in a wide range of common neoplasms and when correctly administered is relatively nonmyelosuppressive (2) but nevertheless effective. This property further enhances its role in drug combinations. An example of such an application is provided by the report of Canellos et al (this issue), where high-dose methotrexate (HDMTX) was incorporated into combination chemotherapy for poor prognosis NHL. Given during the bone marrow recovery phase, ie, 14 days after the

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combination of BACOP, HDMTX effectively prevented tumor regrowth without adversely affecting marrow recovery.

The session on bladder cancer highlights a number of years of clinical experience with MTX in this tumor, which has until recently been considered relatively refractory to chemotherapy. Significant improvements in treatment have resulted from the use of MTX in doses ranging from 100 to 2,000 mg. In particular, the results of Socquet (this issue), if confirmed, will be of considerable importance to urology practice. The addition of MTX therapy to partial cystectomy may render this operation, hitherto considered unsatisfactory, the treatment of choice for invasive bladder cancer.

There has been much debate concerning the possible clinical advantage of massive doses of MTX with FA rescue over lower doses. The controversy concerning the role of HDMTX has, in our opinion, diverted attention from the more important features of the drug: its wide spectrum of activity; its relative lack of acute toxicity when correctly administered; and, in particular, its relative lack of long-term sequelae, most notably the absence of carcinogenesis. Perhaps the attention that has rightly been paid to the evaluation of new agents may have partly eclipsed the existing and potential clinical role of this drug in recent years.

REFERENCES

1. Liefkowitz ER, Papac RJ, and Bertino JR. Head and neck cancer. III. Toxicity of 24 h infusions of methotrexate and protection by leucovorin in patients with epidermoid carcinomas. Cancer Chemother Rep 51:305, 1967.
2. Stoller RG, Hande KR, Jacobs SA, et al. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. N Engl J Med 297:630–634, 1977.
3. Jaffe N, Frei E (iii), Traggis B, et al. Adjuvant methotrexate and citrovorum factor treatment of osteogenic sarcoma. N Engl J Med 291:994–997, 1974.
4. Jackson RC, and Niethanmer B. Acquired methotrexate resistance in lymphoblasts resulting from altered kinetic properties of dihydrofolate reductase. Eur J Cancer 13:567–575, 1975.
5. Shive W, Bardos TJ, Bond TJ, et al. Synthetic members of the folinic acid group. J Am Chem Soc 72:2817–2818, 1950.
6. Bagshawe KD. Lessons from choriocarcinoma. Proc Roy Soc Med 70:303–306, 1977.
7. Bailin PL, Tindall JP, Roenigk HH, et al. Is methotrexate therapy for psoriasis carcinogenic? JAMA 4:359–362, 1975.
8. Canellos GP, DeVita VT, Arseneau JC, et al. Second malignancies complicating Hodgkins disease in remission. Lancet I:947–949, 1975.
9. Walker SE, and Bole, GC. Augmented incidence of neoplasia in female New Zealand black/New Zealand white (NZB–NZW) mice treated with long-term cyclophosphamide. J Lab Clin Med 78:978, 1971.
10. Ross GT. Congenital anomalies among children born of mothers receiving chemotherapy for gestational and trophoblastic neoplasms. Cancer 37:1043–1047, 1976.
11. Li MC, Hertz R, and Spencer DB. Effect of methotrexate therapy upon choriocarcinoma and chorioadenoma. Proc Soc Exp Biol Med 93:361, 1956.
12. Simone JV, Aur RJA, Hustu HO, et al. Total therapy studies of acute lymphocytic leukaemia in children. Cancer 30:1488–1499, 1972.
13. DeVita VT (jr), Serpick AA, and Carbone PP. Combination chemotherapy in the treatment of advanced Hodgkin's disease. Ann Intern Med 73:881–895, 1970.
14. Malpas JS. Childhood solid tumors. Adv Med 13:43–54, 1977.
15. Fraley EE, Lange PH, and Kennedy BJ. Germ cell testicular cancer in adults. N Engl J Med 301:1370–1377 and 1420–1426, 1979.
16. Chapman RM, Sutcliffe SB, and Malpas JS. Cytotoxic-induced ovarian failure in women with Hodgkin's disease. JAMA 242:1877–1881, 1979.
17. Carter SK. The chemical therapy of breast cancer. Semin Oncol 1:131–141, 1974.
18. Carter SK. The chemotherapy of head and neck cancer. Semin Oncol 4:413–424, 1977.
19. Livingstone RB. Treatment of small cell carcinoma. Evolution and future directions. Semin Oncol 5:299–308, 1978.

Recent Concepts on the Mechanism of Action of Methotrexate[1]

J. Courtland White*

The development of cancer treatment regimens that include methotrexate (MTX), especially high-dose plus rescue regimens, has evolved largely from empirical observations in advance of a definitive rationale for their use. It is well known that MTX binds very tightly to its target enzyme, dihydrofolate reductase (DHFR). Blockade of this enzyme prevents regeneration of tetrahydrofolate (H_4folate) from dihydrofolate (H_2folate) produced in the de novo synthesis of thymidylate from uridylate, with the result that pathways that utilize H_4folate cofactors cease to operate—to the extent that exogenous H_4folates are not available or their transport into the cell is blocked by MTX. The inhibition of DHFR by MTX has been described as stoichiometric; that is, one molecule of MTX eliminates one molecule of enzyme. This is essentially the result observed with cell-free extracts at low pH and with folic acid as substrate. At physiological pH, inhibition can best be described as competitive with H_2folate. The K_i is usually quite low, below 10^{-11} M in most cases. It was initially assumed that because of this high-affinity binding, inhibition of H_4folate synthesis and H_4folate-dependent metabolic pathways in the intact cell require only intracellular drug levels near molar equivalence with DHFR. This paradigm also predicts that recovery of a cell from MTX exposure will be slow, being achieved primarily by synthesis of new enzyme. Observations over the past several years that demonstrated rapid recovery of H_4folate-dependent pathways following removal of intracellular MTX in excess of the tightly bound fraction (1–4), and a requirement for free intracellular MTX orders of magnitude above the K_i to maintain inhibition of H_4folate synthesis in vitro (5) have forced a more detailed reconsideration of the mechanism of action of MTX within the intact cell.

REQUIREMENT FOR FREE INTRACELLULAR MTX IN THE INHIBITION OF H_4FOLATE SYNTHESIS

When cells with normal permeability and normal levels of DHFR are first exposed to MTX, the drug penetrates the cell membrane via a carrier-mediated transport system and rapidly binds to the target enzyme. Since transport is rate limiting to binding, initially there is essentially no free intracellular MTX until nearly all DHFR sites are saturated (6,7). Soon after the appearance of "free" MTX, a back flux becomes significant and a steady state for intracellular drug is achieved. If these preloaded cells are transferred to media without MTX, the free drug is rapidly eliminated; however, a portion of intracellular drug indistinguishable from the DHFR level remains (1,3,8). Approximate molar equivalence is maintained. Thus, it was initially surprising to observe that cells prepared in this way were essentially as capable of incorporating ^{3}H-deoxyuridine into DNA as untreated cells (1,3). As shown in figure 1, MTX inhibition of DNA synthesis is a hyperbolic function of the intracellular MTX concentration in excess of the DHFR level with half maximal inhibition in the Ehrlich ascites tumor cell requiring continuous exposure of cells to 2×10^{-7} M MTX (1), a concentration that is more than four orders of magnitude above the DHFR K_i. The requirement for free intracellular MTX is not specific for DNA synthesis, since a similar dose-response relationship is observed for H_4folate-dependent ^{14}C-formate incorporation into RNA, DNA, and protein (2). Furthermore, it was directly demonstrated that free MTX in this range was required to prevent the reduction of ^{3}H-H_2folate to ^{3}H-H_4folate within intact cells (5). Thus, MTX, which is known to have a high affinity for its target enzyme and appears to be permanently bound to DHFR, behaves as a rapidly reversible, low-affinity inhibitor of enzyme function within the cell.

BIOCHEMICAL BASIS FOR REQUIREMENT FOR FREE MTX

Interactions Among MTX, H_2folate, and DHFR Within the Target Cell

Though it is possible that free MTX is required to inhibit an unidentified, low-affinity form of DHFR within the cell or to directly inhibit thymidylate synthetase activity, these explanations, while not necessarily invalid, are not required to explain the above observations. Rather, these observations can be shown to be consistent with the action of MTX at a single high-affinity site (16,17). Reduction of H_2folate

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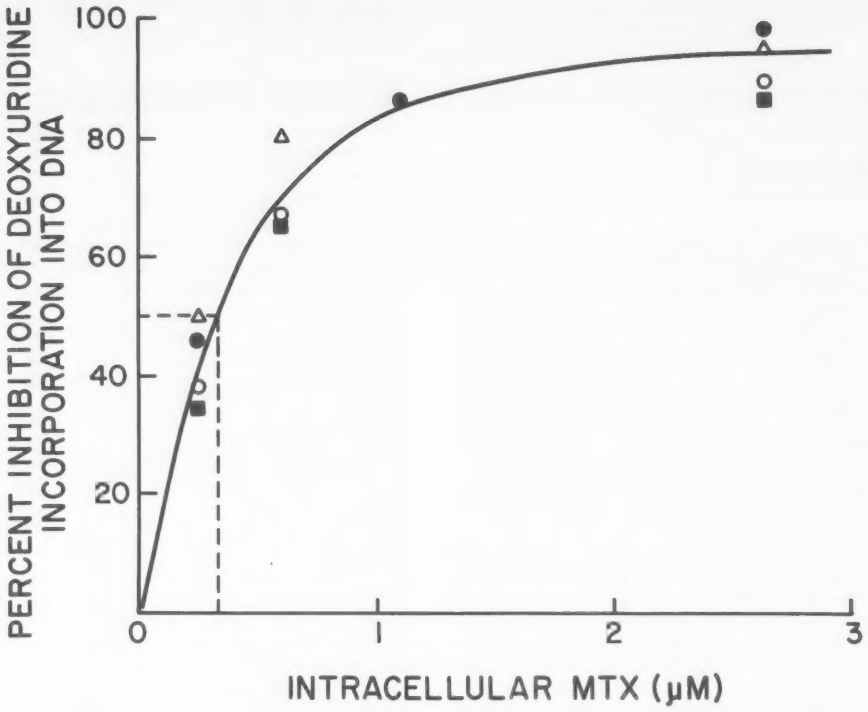

FIGURE 1.—Percent inhibition of deoxyuridine incorporation into DNA by intracellular MTX in excess of the tightly bound fraction. Cells were incubated in media containing a high level of MTX to saturate high-affinity DHFR binding sites, then were transferred to fresh media containing 0 to 6 μM MTX and incubated until a new steady state intracellular MTX concentration was reached. ^{3}H-deoxyuridine was added and rate of incorporation of radiolabel into a trichloroacetic acid precipitate was monitored. The different symbols represent four experiments performed on different days.

is far from being the rate-limiting step in DNA synthesis, since reductase activity is far in excess of the cell's capacity for H_4folate oxidation via thymidylate synthetase (9). H_2folate is converted back to H_4folate as it is formed and the normal cellular concentration of H_2folate is quite low, around 10^{-8} M or less (5,10), with orders of magnitude below the DHFR K_m for this substrate of $\sim 10^{-6}$ M (9,10). Upon exposure of cells to MTX, enzyme sites are rapidly inhibited by MTX. Though total cell DHFR capability is diminished, little effect on H_4folate pools is initially observed since the DHFR that remains is capable of meeting cellular demands for reduced folate. Since cell H_2folate levels are initially low compared to the K_m, the reaction rate is a first order function of the H_2folate concentration. Partial inhibition by MTX, which increases H_2folate levels, serves to increase proportionately the rate of H_2folate reduction at enzyme sites that remain unassociated with MTX. From these considerations, it is clear that depletion of H_4folate cofactor pools by MTX requires inhibition of a large fraction of cellular DHFR. As the intracellular H_2folate concentration approaches and exceeds its DHFR K_m, it competes with MTX for the remaining critical sites. To overcome the competitive effect of H_2folate on MTX binding and to achieve complete inhibition of all of the cell's DHFR activity requires significant concentrations of free intracellular MTX.

Model Studies in a Cell-Free System on the Effects of H_2folate on MTX Binding to DHFR

The large difference in the MTX K_i of $\sim 10^{-11}$ M and the H_2folate K_m of $10^{-6} \sim$ M may make significant competitive interactions between inhibitor and substrate appear unlikely; however, such effects can be demonstrated (10,11). As shown in figure 2, when the concentration of MTX is relatively low, though well

above the MTX K_i, H_2folate can prevent MTX binding to DHFR at concentrations above its K_m (11). At H_2folate concentrations equal to the maximum possible concentration (when all cellular folate has accumulated in this form, ~20–30 μM), prevention of MTX binding is complete. This experiment was carried out at 0 °C to prevent reduction of the competing substrate, H_2folate. At 37 °C, H_2folate, which binds to DHFR in the presence of NADPH, is rapidly reduced to H_4folate, which does not compete with MTX. The ability of H_2folate to prevent MTX binding is, therefore, time dependent. An experiment was designed to stimulate in this cell-free system the time course of events when free MTX has been eliminated from the cell but H_2folate levels are high (fig 3). Pure DHFR was pretreated with excess ^{3}H-MTX and NADPH to saturate all binding sites, then excess free ^{3}H-MTX was separated from the complex. Mixing the complex with excess unlabeled MTX results in a decline in bound ^{3}H-MTX with time, revealing that binding is not irreversible but is characterized by a first order dissociation rate constant. If the ^{3}H-MTX-enzyme complex is mixed instead with H_2folate, a similar pattern of net displacement is initially observed as H_2folate prevents reassociation of ^{3}H-MTX, which dissociates from the enzyme. However, H_2folate that binds to DHFR in the presence of NADPH is rapidly reduced to H_4folate, which does not compete with MTX. Thus, the effect of H_2folate on ^{3}H-MTX binding is time dependent, diminishing as the concentration of H_2folate declines. Eventually, when all H_2folate has been consumed by the enzyme, ^{3}H-MTX binding approaches initial levels.

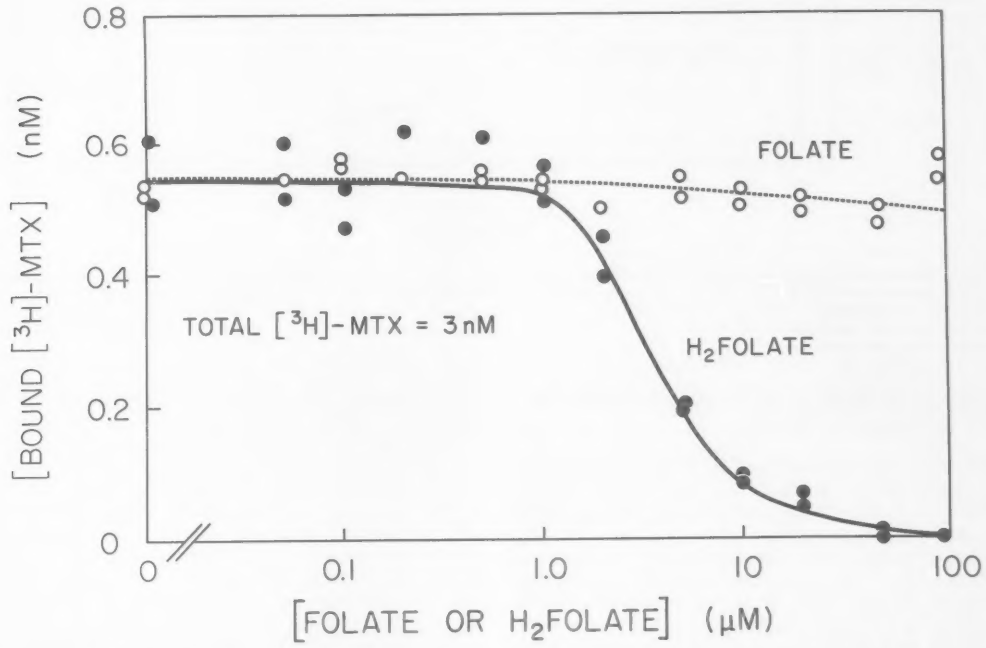

FIGURE 2.—Effect of H_2folate and folic acid on ^{3}H-MTX binding to DHFR at 0 °C. Highly purified enzyme from *Escherichia coli* was pretreated with 100 μM NADPH then added to a mixture of ^{3}H-MTX (3nM) and H_2folate (●) or folic acid (○). After 5 minutes at 0 °C, the mixture was assayed for enzyme-bound ^{3}H-MTX.

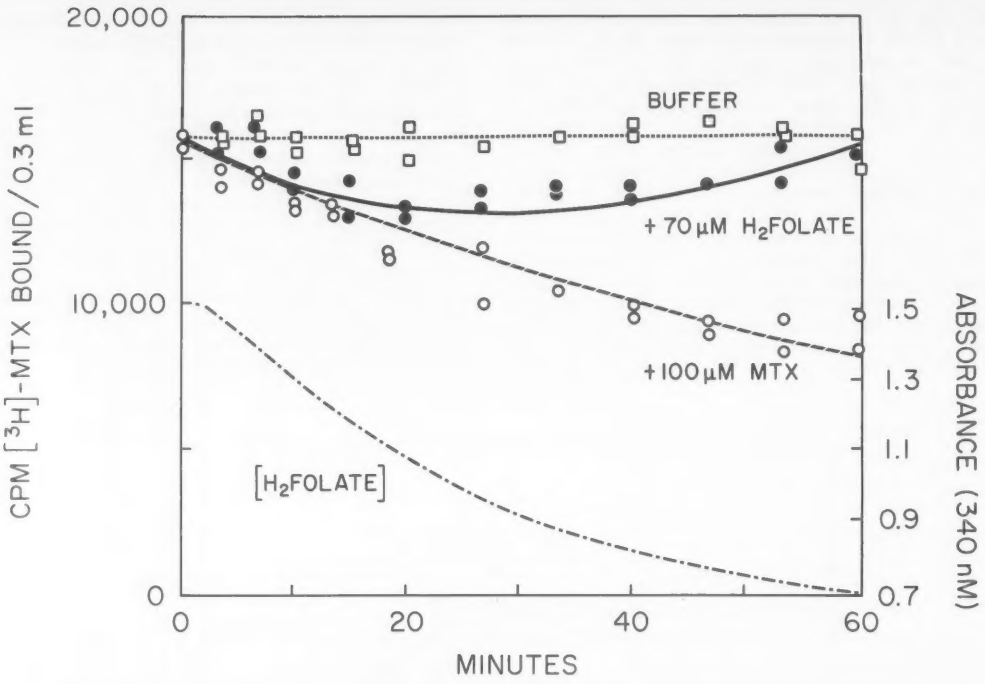

FIGURE 3.—Time course of net displacement of enzyme-bound ^{3}H-MTX by H_2folate and nonlabeled MTX at 37 °C. *E. coli* DHFR was mixed with NADPH and excess ^{3}H-MTX. The complex was separated from free ^{3}H-MTX and diluted 1:1 with buffer (□) or with solutions of unlabeled MTX (final concentration = 100 μM) (○) or H_2folate (70 μM) (●) and assayed at intervals for binding of ^{3}H-MTX. The complex mixed with H_2folate was also incubated at 37 °C in a spectrophotometer and the absorbance at 340 nM, which is proportional to the H_2folate concentration, was monitored.

Computer Analysis of the Interactions Among MTX, H_2folate, and DHFR

The partial displacement of ^{3}H-MTX from binding sites demonstrated in figure 3 cannot be due to the presence of a low-affinity contaminant. First, highly purified, well-characterized enzyme was used for this experiment, and second, the results are exactly those predicted by a computer simulation [2] which considered only experimental- or literature-derived values for MTX and H_2folate binding (fig 4). These values are based on a MTX $K_i = 5 \times 10^{-12}$ M and H_2folate $K_m = 4.4 \times 10^{-7}$ M. The large difference in these constants, five orders of magnitude, is almost the same as the difference between the rate of MTX dissociation, ($k_{off}^{MTX} = 0.01$ minute^{-1}) and the H_2folate turnover rate or the rate of H_4folate production per mole of enzyme ($k_{off}^{H_4\text{folate}} = 1{,}230$ minute^{-1}). It can be directly calculated from these k_{off} values and the K_i and K_m that the rates of binding, k_{on}, of MTX and H_2folate are similar.

By computer simulation it is possible to simultaneously consider numerous factors in the drug-cell interaction and evaluate, among other things, the significance of the competitive substrate versus inhibitor effects described above. Competitive effects are especially apparent and significant when intracellular MTX levels are declining after reaching high levels. The computer was given information on MTX transport, DHFR level, thymidylate synthetase activity, kinetic constants, etc, characteristic of the Ehrlich ascites tumor cell. Figure 5 shows a computer prediction of intracellular events when cells preloaded with excess MTX are transferred to MTX-free media. A similar sequence of events would occur, though on a different time scale, if extracellular MTX declined gradually as from plasma in vivo. Initially, intracellular MTX is high enough to completely inhibit DHFR, H_4folate cofactors are depleted preventing thymidylate synthesis, and H_2folate has accumulated to high levels. As the intracellular MTX level declines to low levels, H_2folate competes with MTX for sites on

[2] The computer simulations were based upon the principles of network thermodynamics and were performed using SPICE2, a computer program for solving the properties of electrical networks. A more complete description of the methods used for computer analysis is provided in reference 11.

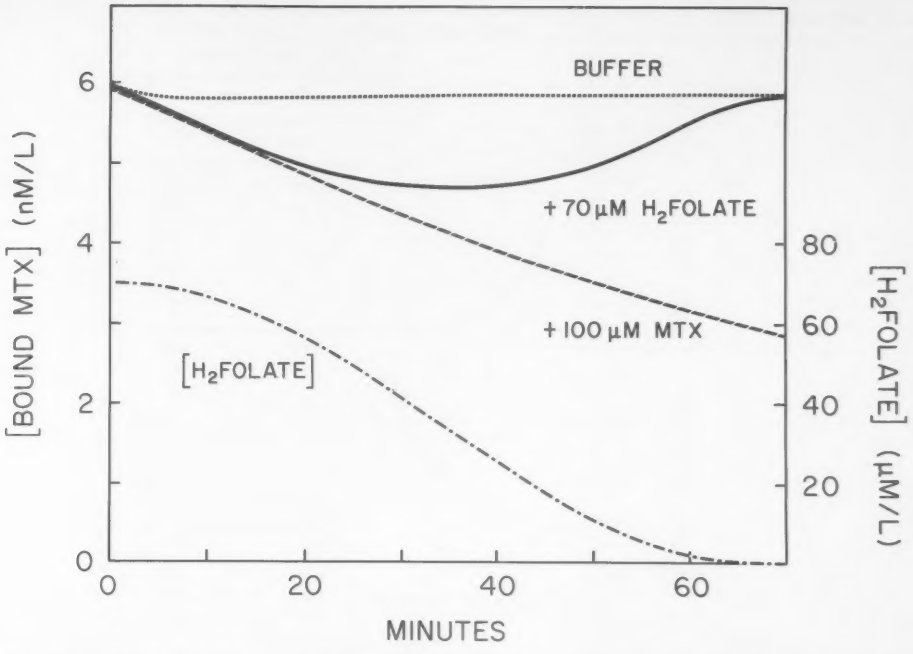

FIGURE 4.—Computer simulation of net displacement of enzyme-bound ^{3}H-MTX. The computer program used the starting conditions described in figure 3 and rate constants that were determined experimentally or from the literature based on a MTX $K_i = 5 \times 10^{-9}$ M and H_2folate $K_m = 4.4 \times 10^{-7}$ M.

DHFR left vacant when bound MTX dissociates. This H_2folate is converted to H_4folate, allowing thymidylate synthesis to resume even before all free MTX has left the cell. Thymidylate synthesis produces H_2folate again; thus, H_2folate levels remain high somewhat longer, but soon the rate of DNA synthesis and the levels of H_2folate and H_4folate are near normal. The lower part of figure 5 examines on an expanded scale the effect of H_2folate on MTX that was bound to high-affinity enzyme sites. If H_2folate is not included in the computer simulation, the decline of bound MTX following removal of free drug is very slow. Though dissociation is occurring, reassociation of MTX with DHFR is much more rapid than drug efflux, and the bound level is essentially constant and equal to the level of total enzyme. If the effect of H_2folate is considered, at the time when free MTX is low but H_2folate is high, H_2folate successfully competes with MTX for available sites on DHFR when bound MTX dissociates. Unlike the previous experiment and simulation in which displaced MTX is confined within the test tube, displaced MTX can migrate out of the cell across the cell membrane. When all or most of the H_2folate is reduced to H_4folate, net displacement of bound MTX is diminished with a portion of intracellular MTX remaining essentially irreversibly bound. This may be very *close* to the level of total enzyme, but is equal to that portion *in excess* of that required to just meet the cell's demand for H_4folate reduction to match the rate of H_4folate oxidation in thymidylate synthesis. Thus, though MTX is indeed a high-affinity inhibitor of DHFR, it *behaves* as a relatively low-affinity, rapidly reversible inhibitor of H_4folate-dependent metabolism because of the large excess of DHFR beyond the cell's minimum needs and the competitive effect of the substrate, H_2folate, which resists inhibition of the last few critical enzyme sites.

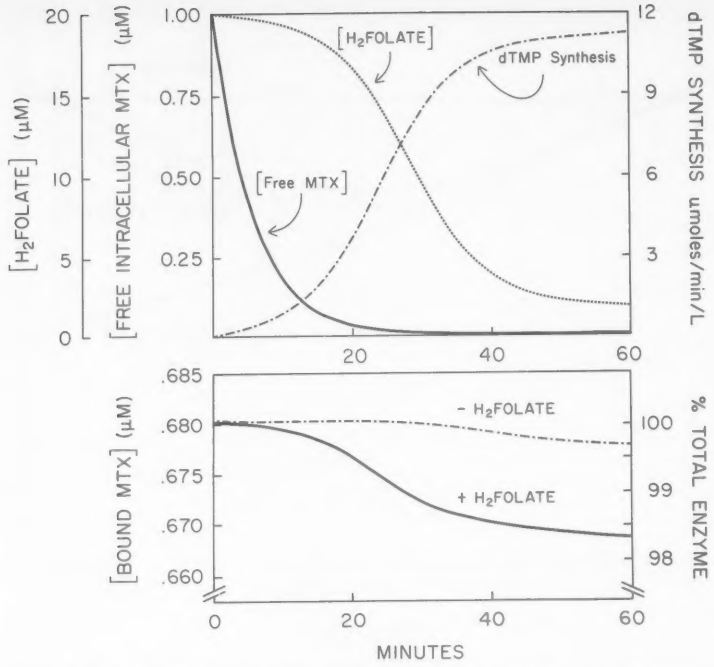

FIGURE 5.—Computer simulation of some cellular events when a cell preloaded with free MTX is transferred to MTX-free media. The computer program used values for DHFR level, H_2folate-H_4folate pool, membrane transport, etc, characteristics of Ehrlich ascites tumor cells.

CYTOTOXIC DETERMINANTS OF MTX

The computer simulation in figure 5 accurately describes at least qualitatively the sequence of events following removal of MTX in any cell. Quantitatively, the sensitivity of a specific cell to MTX depends on a number of the cell's properties. The computer model is and will continue to be a valuable tool for evaluating the importance of potential cytotoxic determinants of MTX cytotoxicity.

Membrane Transport

The properties of the membrane transport system determine not only the rate of entry of MTX into the cell, but also the steady-state free level. As described in more detail by David Goldman (12), the relationship between intracellular and extracellular MTX levels is nonlinear. The intracellular drug concentration approaches a maximum as the extracellular concentration is increased (6). Achievement of high MTX levels inside the cell requires even higher levels outside, perhaps even an order of magnitude higher (13). The achievable MTX concentration may be below the critical level of free drug needed to completely inhibit H_4folate synthesis in certain resistant cell types described below, or the free drug concentration may be lower than normal in cells resistant to MTX by virtue of a transport deficiency. MTX resistance has been associated with decreased transport into the cell (14), and particularly with increases in the influx K_m (13,14), which predicts and correlates with a decrease in the MTX steady state (15) and the rate at which free intracellular drug leaves the cell as the extracellular level declines. Interacuions between MTX and leucovorin (dl-5-formyl-H_4folate) at the level of the membrane carrier may play a key role in the rescue phenomena following high-dose therapy. In addition

to providing reduced folate to bypass the metabolic block imposed by MTX, H_4folate cofactors compete with MTX for transport via their common membrane carrier. A competitive inhibitor has the effect of raising the apparent influx K_m, and since efflux of MTX is not affected, the net effect is a reduction in the steady-state intracellular MTX concentration. This is shown in figure 6, in which tumor cells loaded to a steady state with 1 μM ^{3}H-MTX are treated with natural and unnatural stereospecific preparations of 5-CH_3-H_4folate. The result is almost complete elimination of free intracellular MTX (18,19). As shown in figure 7, this ability of the unnatural H_4folate, which cannot participate in any folate-requiring reaction, to reduce the level of free MTX causes "rescue" or reversal of the inhibitory effect of MTX on DNA synthesis (19).

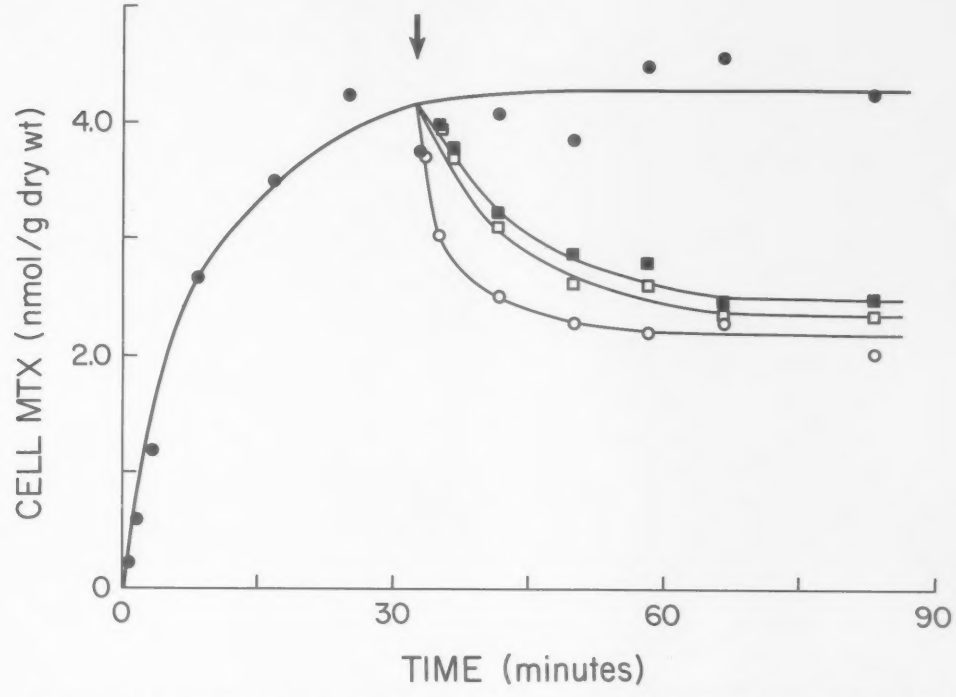

FIGURE 6.—Effect of (−) or (+) CH_3-H_4folate on free intracellular MTX. Cells were exposed to 1.0 μM ^{3}H-MTX and the time course of uptake was measured (●). After 33 minutes (arrow), portions of the cell suspension were exposed to 27 μM (−) or (+) CH_3-H_4folate (■, □, respectively) and net efflux of ^{3}H-MTX was monitored. Another portion of the cell suspension was separated from the media by centrifugation, washed twice with 0 °C buffer, and resuspended in MTX-free medium to determine the level of nonexchangeable intracelluar MTX (o).

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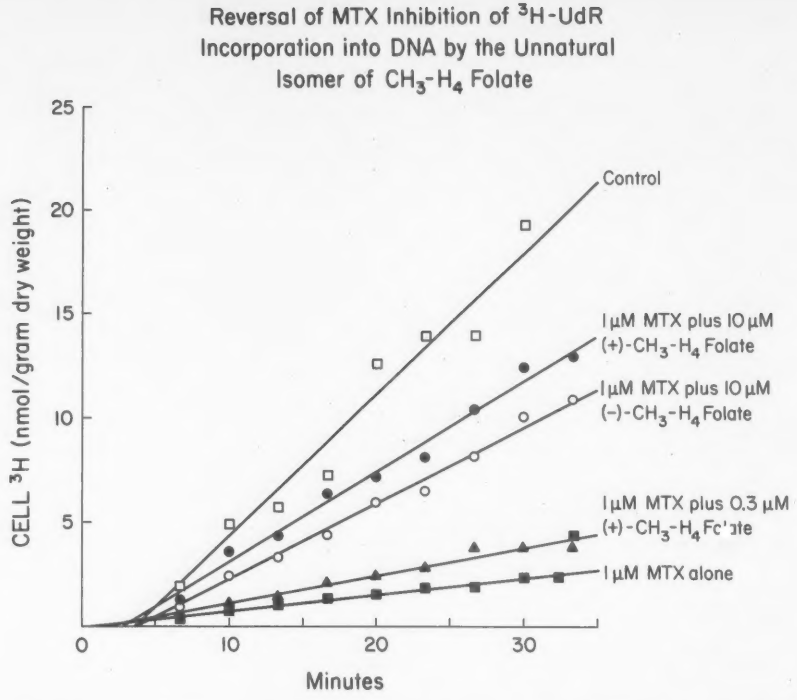

FIGURE 7.—Reversal of MTX inhibition of deoxyuridine incorporation into DNA by (−) or (+) CH_3-H_4folate. Cells were incubated in control media (□) or media containing 1 μM MTX. After 30 minutes, (+) or (−) CH_3-H_4folate (●, ○, respectively) was added to achieve a final concentration of 10 μM or 0.3 μM (+) CH_3-H_4folate (Δ) or no addition was made (■). After 45 minutes, during which time a new steady state for MTX was achieved, ^{3}H-deoxyuridine was added and its incorporation into the TCA precipitate was monitored. From enzymic assay during the preparation of (−) CH_3-H_4folate, it was estimated that 3% of the total CH_3-H_4folate is active (+) isomer. Cells exposed to 0.3 μM (+) CH_3-H_4folate indicated the extent to which this level of the active isomer that accompanies 10 μM (−) CH_3-H_4folate would reverse the MTX block.

The DHFR Level

The most common mechanism of resistance to MTX is an increase in the level of the target enzyme (20). Since DHFR is already in excess, the importance of a further increase may not be obvious. If normally 1 unit of enzyme out of a total of 100 units is sufficient to meet the needs of the cell, then > 99% inhibition by MTX is required to block DNA synthesis. If a resistant cell has a 10-fold increase in DHFR, or 1,000 units, then 99.9% inhibition of total enzyme by MTX is required to begin to inhibit the last critical unit of enzyme activity. This will require approximately a 10-fold increase in free *intracellular* MTX. This may be difficult or impossible to achieve because of properties of the membrane transport system that limit intracellular MTX. Even if complete saturation is achieved, as the extracellular concentration of MTX declines through clearance from plasma, the resistant cell will recover normal rates of DNA synthesis much earlier, even though sufficient free drug is present to prevent H_4folate-dependent metabolism in a normal cell.

The DHFR K_i for MTX

Though binding of MTX to its target site can always be described as tight, the sensitivity of one series of tumor cells has been shown to be a function of the MTX K_i on DHFR. For example, the Yoshida rat sarcoma, whose DHFR K_i of 1.35×10^{-10} M is 25 times greater than that of the L1210 leukemia enzyme of 5.3×10^{-12} M, is 70 times less sensitive to MTX (inhibition of growth) (9). The higher the MTX K_i, the poorer the ability of MTX to interact with DHFR, the more potent the competitive effect of H_2folate, and the greater the level of free drug required to completely suppress H_4folate synthesis.

The Basal Rate of De Novo Thymidylate Synthesis: Effects of Fluoropyrimidines

Thymidylate synthesis is the only known route of oxidation of H_4folate to H_2folate. The rate of this reaction in the cell determines the minimum rate of H_4folate reductase that must be maintained to prevent depletion of H_2folate cofactor pools. If two cells with identical DHFR levels are compared, the one synthesizing DNA and depleting H_4folate pools more rapidly will be more sensitive to MTX. Likewise, a decrease in this rate will increase the fraction of total DHFR that must be inhibited by MTX. Reduction of the basal rate of thymidylate synthesis in Ehrlich ascites cells by prior exposure to fluorodeoxyuridine decreases and at high doses eliminates the ability of MTX to cause a further decline in the rate of DNA synthesis (21). This potential for antagonism of the action of MTX by fluoropyrimidines should be taken into consideration in the scheduling of these agents in combination chemotherapy.

The Synthesis of Polyglutamyl Derivatives of MTX

Conjugation of MTX with glutamic acid to form polyglutamyl derivatives is known to occur at significant rates within a variety of cell types (22–25). These derivatives presumably do not penetrate the cell membrane, or do so slowly, but are fully active as inhibitors of DHFR. Intracellular synthesis of these derivatives to levels in excess of the enzyme-binding capacity results in retention of antifolates and continued inhibition of DNA synthesis, even after clearance of MTX from outside the cell (25). Metabolism of MTX to a nonpermeable form converts MTX from a rapidly reversible to an irreversible inhibitor of DHFR—irreversible to the extent to which MTX polyglutamates are not hydrolyzed back to MTX and are not able to penetrate the cell membrane. The requirement of high levels of free MTX for prolonged periods for synthesis of MTX polyglutamates may be one basis for the efficacy of high-dose MTX regimens.

RATIONALE FOR THE USE OF HIGH-DOSE MTX REGIMENS

These concepts on the reversible nature of the inhibitory effects of MTX within the cell provide a scientific basis for high-dose MTX chemotherapy regimens. Because of the limited ability of cells to generate free MTX and the low fraction of DHFR that is sufficient to meet cellular demand for H_4folate synthesis, very high plasma concentrations may be needed to provide in the tumor cell the intracellular concentration required to achieve the desired pharmacologic effect. These high levels may overcome such properties as impaired membrane transport, high DHFR, high enzyme K_i, and low rate of de novo thymidylate synthesis that confer to tumor cells resistance to conventional low-dose treatment regimens. The high free intracellular MTX levels also may favor synthesis of MTX-polyglutamates that may enhance the extent and duration of antifolate action.

REFERENCES

1. Goldman ID. The mechanism of action of methotrexate. I. Interaction with a low-affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. Mol Pharmacol 10:257–274, 1974.
2. White JC, Loftfield S, and Goldman ID. The mechanism of action of methotrexate. III. Requirement of free intracellular methotrexate for maximal suppression of [^{14}C]formate incorporation into nucleic acids and protein. Mol Pharmacol 11:287–297, 1975.
3. Sirotnak FM, and Donsbach RC. The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. Cancer Res 34:3332–3340, 1974.
4. Jackson RC, and Harrap KR. Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.
5. White JC, and Goldman ID. The mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro. Mol Pharmacol 12:711–719, 1976.
6. Goldman ID, Lichtenstein NS, and Oliverio VT. Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. J Biol Chem 243:5007–5017, 1968.
7. Goldman ID. The characteristics of membrane transport of amethopterin and the naturally occurring folates. Ann NY Acad Sci 186:400–422, 1971.
8. Sirotnak FM, and Donsbach RC. A basis for the difference in toxicity of methotrexate, aminopterin, and methasquin in mice. Biochem Pharmacol 24:156–158, 1975.
9. Jackson RC, Hart LI, and Harrap KR. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductase. Cancer Res 36:1991–1997, 1976.
10. Jackson RC, Niethammer D, and Hart LI. Reactivation of dihydrofolate reductase inhibited by methotrexate or aminopterin. Arch Biochem Biophys 182:648–656, 1977.
11. White JC. Reversal of methotrexate binding to dihydrofolate reductase by dihydrofolate: Studies with pure enzyme and computer modeling using network thermodynamics. J Biol Chem. In press.
12. Goldman ID. Membrane transport considerations in high-dose methotrexate regimens with leucovorin rescue. Cancer Treat Rep 65 (Suppl 1): 13–17, 1981.
13. Goldman ID, Gupta V, White JC, et al. Exchangeable intracellular methotrexate levels in the presence and absence of vincristine at extracellular drug concentrations relevant to those achieved in high-dose methotrexate-folinic acid "rescue" protocols. Cancer Res 36:276–279, 1976.
14. Hill BT, Bailey BD, White JC, et al. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. Cancer Res 39:2440–2446, 1979.
15. Sirotnak FM, and Donsbach RC. Kinetic correlates of methotrexate transport and therapeutic responsiveness in murine tumors. Cancer Res 36:1151–1158, 1976.
16. Goldman ID. Effects of methotrexate on cellular metabolism: some critical elements in the drug-cell interactions. Cancer Treat Rep 61:549–557, 1977.
17. Goldman ID, White JC, and Jackson RC. The concept of methotrexate as a very rapidly reversible inhibitor of dihydrofolate reductase within cells: ramification with respect to MTX-fluoropyrimidine interactions and high dose MTX-leucovorin protocols. *In* Chemistry and Biology of Pteridines (Kisliuk RL, and Brown GM, eds). New York, NY, Elsevier/North Holland, 1979, pp 625–627.
18. White JC, Bailey BD, and Goldman ID. Lack of stereospecificity at carbon 6 of methyltetrahydrofolate transport in Ehrlich ascites tumor cells. J Biol Chem 253:242–245, 1978.

19. White JC, and Goldman ID. Lack of stereospecificity at carbon 6 of methyltetrahydrofolate transport: possible relevance to rescue regimens with methotrexate and leucovorin. *In* Chemistry and Biology of Pteridines (Kisliuk RL, and Brown GM, eds). New York, NY, Elsevier/North Holland, 1979, pp 625–627.
20. Neithammer D, and Jackson RC. Changes of molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cell. Eur J Cancer 11:845–854, 1975.
21. Bowen D, White JC, and Goldman ID. A basis for fluoropyrimidine-induced antagonism to methotrexate in Ehrlich ascites tumor cells in vitro. Cancer Res 38:219–222, 1979.
22. Gewirtz DA, White JC, Randolph JK, et al. Formation of methotrexate polyglutamates in rat hepatocytes. Cancer Res 39:2914–2918, 1979.
23. Gewirtz DA, White JC, Randolph JK, et al. Membrane transport, binding and polyglutamation of the folic acid analog, methotrexate, in freshly isolated rat hepatocytes. Cancer Res. In press.
24. Whitehead MV, Perrault MM, and Stelcner S. Tissue-specific synthesis of methotrexate polyglutamates in the rat. Cancer Res 35:2985–2990, 1975.
25. Rosenblatt DS, Whitehead VM, Vera N, et al. Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamates by cultured human cells. Mol Pharmacol 14:1143–1147, 1978.

Membrane Transport Considerations in High-Dose Methotrexate Regimens with Leucovorin Rescue

I. David Goldman*

While the potential role of membrane transport of antifolates in cancer chemotherapy was recognized early, the basis for a clear link between membrane transport and the ability of these agents to achieve their pharmacologic effect has come only recently (1–4). These insights derive from a comprehensive analysis of the interaction between the drug and mammalian cells, encompassing not only membrane transport, but a highly quantitative evaluation of the interaction among methotrexate (MTX), dihydrofolate reductase (DHFR), and dihydrofolate within the intact cell (5–8). White (9) has described the critical role for free intracellular MTX in the suppression of tetrahydrofolate synthesis, and has identified a variety of factors that increase the requirement for this drug component to achieve a pharmacologic effect. These elements, along with the properties of the transport system that limit the accumulation of free intracellular MTX, as described in this paper, may provide, in part, a rationale for high-dose MTX protocols.

Membrane transport is often visualized as the penetration of an agent into the cell, a one-way flux across the cell membrane. For an anticancer agent, it might be expected that the more rapid the flux into the tumor cell relative to the flux into susceptible host tissues, the more nearly ideal the therapeutic index. While it is a very restricted view of transport, this might, in fact, be relevant for chemotherapeutic agents that interact irreversibly with their intracellular targets and for which the flux into the cell is rate limiting to the subsequent reaction between drug and target. Accordingly, if transport is sufficiently slow, a critical inactivation of the target site may not be achieved over the interval of exposure to the drug to produce irreversible cell damage. However, once the drug reaches molar equivalence with its target site, the target should become functionally inert. A more complicated picture of the drug-cell interaction controlled by the membrane transport system emerges with an anticancer agent that is a reversible inhibitor of its target site, as is the case for MTX. As described in detail in White's paper (9), while the MTX K_i for DHFR reductase is very low, the pharmacologic effect of MTX is rapidly reversible because of the low fraction of total enzyme that is sufficient to sustain cellular tetrahydrofolate synthesis at normal rates, and the competitive interaction among dihydrofolate, MTX, and DHFR as the major portion of the target enzyme becomes associated with the drug (2,5–8). For a reversible inhibitor to achieve its pharmacologic effect, particularly if this requires association with the major portion of the target sites, substantial levels of free drug in comparison to the drug K_i are required. Hence, the critical element in this drug-target interaction is the level of free drug that accumulates within the intracellular water and the duration over which the free drug is sustained following its administration.

In relating elements of the membrane transport of MTX to its pharmacologic activity, a number of factors must be considered. The flux of MTX into the cell (influx) is clearly important, since influx is rate limiting to binding of MTX to the bulk of the DHFR sites (10). If influx is sufficiently slow, inactivation of enzyme may proceed at a slower rate than the rate of synthesis of new enzyme. When it is considered that more than 95% of DHFR must be associated with MTX before tetrahydrofolate synthesis is reduced (11), this pharmacologic endpoint may not be achieved. Influx, per se, is also important because at low drug concentrations influx properties correlate well with the ability of cells to achieve and maintain a transmembrane gradient for this agent (1), and it is the control of this intracellular MTX component that is the focus of this article.

FACTORS THAT INFLUENCE ACCUMULATION AND RETENTION OF FREE INTRACELLULAR MTX

The Transmembrane Electrical-Potential Difference

Consideration of transmembrane gradients for MTX is complicated because the drug is a bivalent anion and mammalian cells carry a net negative charge (10). Transmembrane gradients cannot be considered in terms of chemical gradients alone; rather, with respect to the energetics of transport, the critical factor is the electrochemical-potential difference for the drug across the cell membrane. In other words, since the cell and drug carry a negative charge, if the steady-state level of intracellular drug

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achieved is at equilibrium with extracellular drug (ie, if transport is passive and not energy dependent), the electrical force of repulsion tending to drive the negatively charged MTX out of the negatively charged cell must be exactly equal to the chemiosmotic force tending to drive the drug into the cell. Hence, at equilibrium, the concentration of MTX in the intracellular water must be less than its extracellular concentration, the extent of which will depend upon the magnitude of the cell electronegativity. For instance, when the membrane potential is 15 mv, the ratio of free intracellular to extracellular MTX at equilibrium will be 0.3; if the membrane potential is 30 mv, this ratio is reduced to 0.1; and when the membrane potential is 45 mv, this ratio is reduced to 0.01. Or, if the membrane potential is 45 mv when the extracellular MTX is 1 μM, the free intracellular MTX level for a passive system will be 30 nM. When extracellular MTX is 10 μM under these conditions, the intracellular MTX level will be 3.0 μM, and when the extracellular drug level is 1 mM, the intracellular drug level will only be 30 μM. This means that there is an important electrical force that limits the accumulation of free intracellular MTX and governs the distribution of MTX across the cell membrane, under conditions in which the major portion of MTX transport is influenced by either passive diffusion or some non-energy-dependent carrier system. Further, this electrical element will influence the final chemical gradient for MTX achieved even in the presence of an active transport system and, in either case, must be considered in evaluating the presence or absence of active (energy-dependent) transport for this agent.

The Relationship Between Influx and Efflux Properties for MTX; Ramifications in Terms of the Accumulation of Free Intracellular Drug

Biological membranes permit bidirectional flows of substances across this interface. While it is possible to have channels or pores that would permit a one-way flux alone, this would be the exception and not the rule. The level of MTX within the intracellular water must be considered in the net effect of these bidirectional flows. The evidence at the present time indicates that there is a membrane transport carrier that facilitates the translocation of MTX into and out of many mammalian cells. This transport system is normally used for transport of endogenous tetrahydrofolate cofactors, but can be parasitized by MTX. Transport by this route is a cyclic phenomenon that achieves the rapid translocation of these hydrophilic compounds through the lipid cell membrane (12). At low MTX levels, influx is accounted for entirely on the basis of this single saturable mechanism. While it is possible that there is, in addition, a second transport route that contributes significantly to MTX influx at higher extracellular drug levels, and/or a second route for MTX efflux that may mediate transport of folic acid out of the cell as well, this issue is not yet resolved (13). If a carrier acts on the substrate that it transports symmetrically, ie, the nature of the interaction between the substrate and its carrier as it moves into the cell is the same as the interaction between the substrate and the carrier as they move out of the cell, and if the mobilities of the loaded and unloaded carriers as they move into and out of the cells are symmetrical (although the mobility of the loaded and unloaded carriers need not be the same), then this is, by definition, a system that can achieve no gradients and is independent of cellular energy metabolism. If, however, there is an asymmetry in this system so that the flow of substrate in one direction is different from the flow of substrate in the opposite direction, then this will generate a gradient for the substrate across the cell membrane. This asymmetry must be accounted for on the basis of some expenditure of energy such as the hydrolysis of ATP or an interaction between the transport system and some cation or other substance asymmetrically distributed across the cell membrane (1).

There is considerable evidence to indicate that MTX transport in a variety of mammalian cells is asymmetrical. The apparent affinity of the transport system as it mediates the MTX flux into the cell is much greater than the affinity of the transport system as it mediates the flux of MTX out of the cell (4,10). Indeed, the K_m for MTX efflux may be so high that the process may not demonstrate saturability at all. (The K_m for influx is the extracellular level of substrate at which the transport system is half saturated. The higher the affinity of the transport carrier for its substrate, the lower the extracellular concentration at which the transport system saturates and vice versa.) This asymmetry in the kinetics of influx and efflux predicts that the cells should sustain transmembrane gradients for the drug. This is, in fact, the case (10). When the transmembrane electrical-potential difference for methotrexate is considered, the actual concentration of free intracellular MTX exceeds that expected for equilibrium, compatible with an "active transport" mechanism; that is, energy is required for transport of MTX into the cell. There are a number of important ramifications of this kind of asymmetrical transport system. First, the transmembrane gradient achieved for MTX is dependent on the extracellular drug concentration. When the drug level is low, the gradient is high; when the extracellular drug concentration is high, the gradient is low. Indeed, when the transport carrier is the sole route for translocation of drug into and out of the cell, then as the extracellular concentration approaches infinity, the intracellular drug concentration approaches some maximum value and the ratio of intracellular to extracellular drug falls towards zero (1). For many mammalian cells, the maximum level of free intracellular MTX that can be achieved by the transport system is in the range of 5 μM (4,10). Hence, while at low drug levels the transport system is concentrative; paradoxically, at high drug levels the membrane transport system restricts accumulation of free drug within the intracellular

compartment (1). If, on the other hand, there is an additional carrier system available or passive diffusion becomes a prominent component of the bidirectional fluxes at high extracellular levels, then the concentration of free intracellular drug that can be achieved will be in excess of that predicted for the asymmetrical carrier system. While the free intracellular MTX level does exceed that predicted for the carrier system at high extracellular MTX levels, the magnitude of this difference is small and the ratio of free intracellular to extracellular MTX remains quite low (14). This is not unexpected since, when the distribution of MTX is determined by passive factors alone, the ratio of intracellular to extracellular drug will be governed by the membrane potential; and, as indicated above, when the membrane potential is high, this ratio will be extremely low.

Energy-Dependent Processes Directly Limit Accumulation of Free Intracellular MTX

Free intracellular MTX levels, as above mentioned, are limited by electrostatic forces as well as an asymmetrical transport carrier system. In addition, energy is coupled either to this carrier system or to an independent exit mechanism to limit the accumulation of free intracellular drug as suggested by the observation that a variety of metabolic poisons augment rather than retard the free intracellular MTX level (15). While this represents yet another element restricting the accumulation of free intracellular MTX, selective inhibition of this process may be a useful approach to MTX chemotherapy. For instance, vinca alkaloids appear to block this mechanism (16) by inhibition of cellular energy metabolism (17), at least as it affects membrane transport processes (18), resulting in increased MTX inhibition of DNA synthesis in vitro (6). Vincristine also synergizes with MTX in vivo (16,19), an effect that may be related to enhanced free intracellular MTX levels, although this is not, as yet, resolved. Enhanced chemotherapeutic efficacy in vivo suggests selectivity and raises the possibility that vincristine-MTX interactions in this context may be quantitatively different in tumor cells versus susceptible host tissues.

Hence, a variety of passive and active factors limits the accumulation of free intracellular MTX, the drug component that is required for inhibition of tetrahydrofolate synthesis.

INTERACTIONS BETWEEN LEUCOVORIN AND MTX AT THE LEVEL OF THE MEMBRANE TRANSPORT SYSTEM IN RESCUE REGIMENS

MTX is transported via the carrier system usually present for tetrahydrofolate cofactors (12). A variety of competitive interactions at the level of the transport carrier can be demonstrated for these substances (10,12,20). For instance, extracellular MTX will inhibit entry of tetrahydrofolate cofactors into the cell and should reduce the level of free intracellular tetrahydrofolate cofactor monoglutamates. Conversely, extracellular tetrahydrofolate cofactors will inhibit MTX influx into cells and reduce the exchangeable intracellular MTX level (10,12). The ratio of the concentrations of these compounds in the extracellular compartment and the relationship between these concentrations and the affinities of the carrier for these substrates will determine the extent to which MTX and tetrahydrofolate cofactors can perturb each other's transport. Based upon this consideration, described in more detail elsewhere (21), some generalizations are possible: (a) During high-dose MTX administration not only is endogenous tetrahydrofolate synthesis abolished, but MTX inhibition of methyltetrahydrofolate influx may be so great that this circulating cofactor may not be available for cellular macromolecular synthesis. (b) Immediately after high-dose MTX administration, low levels of leucovorin (LV) may be superfluous, since the high extracellular MTX concentration may markedly inhibit LV transport into cells. (c) Analysis of membrane transport interactions provides clarification of the mechanism of rescue under certain conditions. LV can rescue by delivery of LV into the cell, circumventing the inhibition of tetrahydrofolate synthesis by MTX. In addition, however, LV has the capacity to reverse the pharmacologic effect of MTX by blocking its influx, resulting in efflux of free intracellular MTX with the resumption of endogenous cellular tetrahydrofolate synthesis. With low-dose LV, rescue is likely due to the delivery and utilization of LV substrate, bypassing the metabolic block induced by MTX. Hence, in many regimens, blood levels of MTX, when LV is administered, should provide a sufficiently high concentration of free intracellular MTX to block tetrahydrofolate synthesis, but an insufficient level to reduce LV entry into the cell to a level limiting cellular biosynthetic processes and replication. Likewise, the extracellular LV level achieved in most rescue regimens that is sufficient to rescue cells will not result in sufficient egress of free intracellular MTX to reverse the pharmacologic effect of this agent. However, when high doses of LV are administered, this may drive enough free MTX out of the cell to permit resumption of cellular tetrahydrofolate synthesis. Hence, high-dose LV rescue offers the opportunity for delivering high levels of LV into the cell and the potential for eliminating the pharmacologic effect of MTX as well, particularly when there is a failure to excrete MTX normally and high MTX blood levels are sustained. There remains a host of unanswered questions regarding this interaction, many of which are quantitative in nature. The extent to which these interactions occur in vivo requires determination of transport and inhibitor constants for MTX and tetrahydrofolate cofactors in tumor cells and susceptible host tissues. It is unclear what rates of LV delivery into the cell are required to meet cellular demands for tetrahydrofolate cofactors under conditions in which regeneration of tetrahydrofolate from dihydro-

folate is abolished. Under normal conditions, a net increase in cellular tetrahydrofolate is necessary only to maintain cofactor pools during cell replication. Although the rate of tetrahydrofolate oxidation to dihydrofolate is high, resynthesis is sufficiently fast so that tetrahydrofolate cofactor pools are maintained. When this cycle is blocked, cell dihydrofolate pools increase rapidly and tetrahydrofolate-dependent processes depend entirely upon delivery of extracellular tetrahydrofolate cofactors into the cell. During rescue, there must be sufficient LV in the extracellular compartment to overcome the inhibitory effect of MTX on transport and to deliver that critical level of LV into the cell to meet the cellular need for tetrahydrofolate in macromolecular synthesis.

ADMINISTRATION OF MTX AS A BOLUS VERSUS AN INTRAVENOUS INFUSION

There are a number important differences between high-dose MTX regimens in which the drug is given as a bolus versus a sustained infusion. For instance, if the drug is administered as a bolus, then the total interval over which a critical level of free intracellular MTX is sustained depends upon the initial level of free intracellular MTX achieved and the rate of decline of free intracellular drug as the extracellular concentration falls. Since the former is very limited, the latter may be crucial and, indeed, the cytotoxicity of MTX in a variety of animal tumors is related to the rate of decline of the free intracellular MTX level (4). This may be a basis for MTX selectivity in the mouse, in that the rate of decline of free intracellular drug from cells of the small intestine is much more rapid than from tumor cells (3). Conversely, when the drug is infused over long intervals, the duration over which free drug levels decline after cessation of the infusion may be small in comparison to the duration of the infusion and it may be the absolute level of drug achieved during the infusion that is more important than the rate of decline following the infusion. Properties of the MTX transport system that determine the free intracellular drug level differ depending upon the mode of administration and blood level of MTX achieved in these protocols. With pulse administration, the property of the transport system that best correlates with the decline of free intracellular drug is the influx K_m, a parameter of the affinity of the transport system for MTX (4). On the other hand, during drug infusion, the free intracellular MTX level achieved may be determined by membrane charge (see above) as well as properties of the transport system. When the extracellular concentration is near the K_m for the transport carrier, then the influx K_m will influence the free MTX level achieved. However, when the extracellular MTX concentration is high in comparison to the K_m, then this parameter no longer influences the level of MTX achieved within the cell. Rather, a critical parameter becomes the transport maximum for the carrier system—the maximum rate of cycling of the carrier and the maximum number of carrier sites within the membrane. With high-dose pulse administration of MTX, it is only when the extracellular drug level falls within the range of the carrier K_m that this parameter becomes an important factor in determining the rate of drug loss from the cell.

HIGH-DOSE MTX AND THE SOLID TUMOR

Up to this point, the rationale for high-dose MTX protocols has been discussed within the context of the requirement for substantial levels of free intracellular drug to inhibit tetrahydrofolate synthesis. Another factor may be the diffusion of drug into large tumor masses. Clearly, as the tumor cell distance from the blood supply increases, the availability of drug to the tumor cell decreases. High-dose regimens increase net diffusion of drug to potential pharmacologic sanctuaries such as the center of a tumor mass. Likewise, this consideration may be relevant to selectivity in that diffusion of MTX out of a tumor mass as the extracellular blood level falls should be much slower than diffusion from well-perfused tissues such as bone marrow or gastrointestinal epithelium. Further, this could also be a basis for selectivity in low-dose LV rescue in that LV would be rapidly delivered to host renewal tissues but would diffuse slowly into tumor masses that retain high levels of MTX and do not synthesize tetrahydrofolate.

MTX POLYGLUTAMATES AND THEIR POTENTIAL ROLE IN DRUG CYTOTOXICITY AND SELECTIVITY

MTX is a rapidly reversible inhibitor of tetrahydrofolate synthesis by virtue of the rapid efflux of free drug from the cell as the extracellular level falls. The conversion of MTX to MTX polyglutamate derivatives now reported for many tissues is potentially of considerable importance (22–26). It is likely that polyglutamate derivatives of naturally occurring tetrahydrofolates as well as dihydrofolate are retained within cells and in some cases have enhanced cofactor potency in comparison to their monoglutamates (27–29). Indeed, the loss of the ability of cells to form polyglutamates of tetrahydrofolate cofactors is a lethal mutation in the absence of nucleoside and glycine (30,31). Similarly, addition of glutamate moieties to MTX does not alter MTX binding to DHFR reductase; in fact, binding may be enhanced (32). It would be expected that these polyglutamates of MTX are retained within the cell and, indeed, a transmembrane gradient for MTX polyglutamates has been observed in the rat hepatocyte (33). It has been suggested, however, that polyglutamates of MTX rapidly exit from L1210 leukemia cells (34). The issue, while not

resolved, is important because these MTX derivatives have the capacity to prolong inhibition of DHFR to the extent that the polyglutamates are not hydrolyzed to monoglutamates and are retained within the intracellular compartment. Recently, studies with human fibroblasts have correlated accumulation of MTX polyglutamates with sustained inhibition of deoxyuridine incorporation into DNA (35). Finally, the ability of host renewal tissues versus tumor to form and retain MTX polyglutamates could play an important role in drug selectivity.

REFERENCES

1. Goldman ID. Uptake of drugs and resistance. *In* Drug Resistance and Selectivity: Biochemical and Cellular Basis (Mihich E, ed). New York, NY, Academic Press, Inc, 1973, vol 1, chap 8, pp 299–358.
2. Goldman ID. Effects of methotrexate on cellular metabolism: some critical elements in the drug-cell interaction. Cancer Treat Rep 61: 549–558, 1977.
3. Sirotnak FM, and Donsbach RC. Further evidence for a basis of selective activity and relative responsiveness during antifolate therapy of murine tumors. Cancer Res 35:1737–1744, 1975.
4. Sirotnak FM, and Donsbach RC. Kinetic correlates of methotrexate transport and therapeutic responsiveness in murine tumors. Cancer Res 36:1151–1158, 1976.
5. Goldman ID. The mechanism of action of methotrexate I. Interaction with a low-affinity intracelluar site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. Mol Pharmacol 10:257–274, 1974.
6. Goldman ID, and Fyfe MJ. The mechanism of action of methotrexate II. Augmentation by vincristine of inhibition of DNA synthesis by methotrexate in Ehrlich ascites tumor cells. Mol Pharmocol 10:275–282, 1974.
7. White JC, Loftfield S, and Goldman ID. The mechanism of action of methotrexate III. Requirement of free intracellular methotrexate for maximal suppression of [^{14}C] formate incorporation into nucleic acids and protein. Mol Pharmacol 11:287–297, 1975.
8. White JC, and Goldman ID. The mechanism of action of methotrexate IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro. Mol Pharmacol 12:711–719, 1976.
9. White JC. Recent concepts on the mechanism of action of methotrexate. Cancer Treat Rep 65 (Suppl 1):3–12, 1981.
10. Goldman ID, Lichtenstein NS, and Oliverio VT. Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. J Biol Chem 243:5007–5017, 1968.
11. Jackson RC, and Harrap KR. Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.
12. Goldman ID. The characteristics of the membrane transport of amethopterin and the naturally occurring folates. Ann NY Acad Sci 186:400–422, 1971.
13. Hill BT, Bailey BD, White JC, et al. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. Cancer Res 39:2440–2446, 1979.
14. Goldman ID, Gupta V, White JC, et al. Exchangeable intracellular methotrexate levels in the presence and absence of vincristine at extracellular drug concentrations relevant to those achieved in high-dose methotrexate-folinic acid "rescue" protocols. Cancer Res 36:276–279, 1976.
15. Goldman ID, Transport energetics of the folic acid analogue, methotrexate, in L1210 leukemia cells. J Biol Chem 244:3779–3785, 1969.
16. Zager RF, Frisby SA, and Oliverio VT. The effects of antibiotics and cancer chemotherapeutic agents on the cellular transport and antitumor activity of methotrexate in L1210 murine leukemia. Cancer Res 33:1670, 1973.
17. Fyfe MJ, and Goldman ID. Characteristics of the vincristine-induced augmentation of methotrexate uptake in Ehrlich ascites tumor cells. J Biol Chem 248:5067–5073, 1973.
18. Fyfe MJ, Loftfield S, and Goldman ID. A reduction in energy-dependent amino acid transport by microtubular inhibitors in Ehrlich ascites tumor cells. J Cell Physiol 86:201–211, 1975.
19. Chello PL, Sirotnak FM, and Dorick DM. Different effects of vincristine on methotrexate uptake by L1210 cells and mouse intestinal epithelia in vitro and in vivo. Cancer Res 39:2106–2112, 1979.
20. Goldman ID. The model system for the study of heteroexchange diffusion: methotrexate-folate interactions in L1210 leukemia and Ehrlich ascites tumor cells. Biochim Biophys Acta 233:624–634, 1971.
21. Goldman ID. Membrane transport of methotrexate (NSC-740) and other folate compounds: relevance to rescue protocols. Cancer Chemother Rep 6:63–72, 1975.
22. Brown JP, Davidson GE, Weir DG, et al. Specificity of folate-γ-L-glutamate ligase in rat liver and kidney. Biosynthesis of poly-γ-L-glutamates of unreduced methotrexate and the effect of methotrexate on folate polyglutamate biosynthesis. Int J Biochem 5:727–733, 1974.
23. Rosenblatt DS, Whitehead VM, DuPont MM, et al. Synthesis of methotrexate polyglutamates in cultured human cells. Mol Pharmacol 14:210–214, 1978.
24. Whitehead VM. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. Cancer Res 37:408–412, 1977.
25. Whitehead VM, Perrault MM, and Stelcner S. Tissue-specific synthesis of methotrexate polyglutamates in the rat. Cancer Res 35:2985–2990, 1975.
26. Jacobs SA, Derr CJ, and Johns DG. Accumulation of methotrexate diglutamate in human liver during methotrexate therapy. Biochem Pharmacol 26:2310–2313, 1977.
27. Cheng FW, Shane B, and Stokstad ELR. Pentaglutamate derivatives of folate as substrates for rat liver tetrahydropteroylglutamate methyltransferase and 5,10-methylenetetrahydrofolate reductase. Can J Biochem 53:1020–1027, 1975.
28. Coward JK, Parameswaran KN, Cashmore AR, et al. 7,8-dihydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with purified dihydrofolate reductase from mammalian sources. Biochemistry 13:3899–3903, 1974.
29. Coward JK, Chello PL, Cashmore AR, et al. 5-methyl-5,6,7,8 tetrahydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with methionine synthetase from bovine brain. Biochemistry 14:1548–1552, 1975.
30. Taylor RT, and Hanna ML. Folate-dependent enzymes in cultured Chinese hamster cells: folypolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. Arch Biochem Biophys 181:331–334, 1977.
31. McBurney MW, and Whitmore GF. Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. Cell 2:173–182, 1974.
32. Jacobs SA, Adamson RH, Chabner BA, et al. Stoichiometric inhibition of mammalian dihydrofolate reductase by the γ-glutamyl metabolite of methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-glutamate. Biochem Biophys Res Commun 63:692–698, 1975.
33. Gewirtz DA, White JC, Randolph JK, et al. Formation of methotrexate polyglutamates in rat hepatocytes. Cancer Res 39:2914–2918, 1979.
34. Sirotnak FM, Chello PL, Piper JR, et al. Growth inhibitory, transport and biochemical properties of the γ-glutamyl and γ-aspartyl peptides of methotrexate in L1210 leukemia cells in vitro. Biochem Pharmacol 27:1821–1825, 1978.
35. Rosenblatt DS, Whitehead VM, Vera N, et al. Prolonged inhibition of DNA synthesis associated with the accumulation of methotrexate polyglutamates by cultured human cells. Mol Pharmacol 14:1143–1147, 1978.

Role of Membrance Transport in the Pharmacologic Action of Antifolates [1,2]

F. M. Sirotnak*

SUMMARY

The property of accumulating and maintaining pharmacologically effective intracellular levels of folate analogs is different among proliferative tissues, both normal and tumorous, in mice. The higher sensitivity of the small intestine compared to bone marrow for folate analogs is related to greater retention of unbound drug and more prolonged inhibition of DNA synthesis in the former. Similar differences favoring greater retention of drug and more prolonged inhibition of DNA synthesis in responsive tumors compared to nonresponsive tumors and small intestine appear to account for the selective antitumor action of these agents in murine tumor models. The observed pharmacokinetic differences among tissues are largely attributable to differences in the structural specificity of carrier-mediated membrane transport of folate analogs.

[Cancer Treat Rep 65 (Suppl. 1):19–27, 1981]

CORRELATES OF ANTIFOLATE TRANSPORT AND CYTOTOXICITY

Most folate analogs mediate their cytotoxic effects through the inhibition of dihydrofolate reductase (DHFR) (1,2). The inhibition results in a depletion of the tetrahydrofolate pool with a consequent cessation in dTMP synthesis and, ultimately, purine and amino acid synthesis. The magnitude of the cytotoxicity observed in each tissue is a function of both the extent and duration of the inhibition of DNA synthesis (3–9), the primary metabolic effect of the drug-enzyme interaction. The period during which complete inhibition of DHFR is sustained appears to be determined by the properties of the transport system in each tissue. The accumulation of DHFR unbound antifolate appears necessary to initiate and sustain inhibition of DNA synthesis. Studies in vivo show the rapidly reversible nature of the drug-induced inhibition of DNA synthesis in mouse small intestine (3,4), and an extracellular concentration dependence for resumption of [^{3}H] UdR incorporation in L1210 cells, small intestine, and bone marrow (6). In vivo studies (5,7,8) provided direct evidence associating the decrease in intracellular unbound methotrexate (MTX) with a resumption in DNA synthesis in both small intestine and tumor cells. Studies in vitro with resting cell suspensions (7,10,11) also provide evidence for a relationship between the level of intracellular exchangeable MTX and the extent of suppression of DNA, RNA, and folate-H_4 synthesis. Free levels of intracellular antifolate are necessary to fully saturate DHFR (7,10,11). This is related (7,10,11) to the partially reversible nature of the binding of antifolate to this enzyme (first described by Werkheiser [1]), to the increased competition for reversible binding of antifolate (11) resulting from the accumulation of dihydrofolate during dTMP synthesis, and to excess DHFR above what is actually needed to maintain DNA synthesis (7,12). In agreement with the theoretical considerations of Goldman (13), our experimental evidence (5,7,8,14) supports the hypothesis that the critical determinant of cytotoxicity of antifolates is the intracellular level of unbound drug.

RELATIVE RESPONSIVENESS AND SELECTIVE ANTITUMOR ACTION

A general correlation between the extent of uptake of MTX by a group of murine leukemias in vitro and

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[2] Methotrexate: NSC-740, supplied by the Drug Development Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI.

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their therapeutic responsiveness in vivo was first reported by Kessel and coworkers (16). These workers found a linear relationship over a fourfold range between the extent of uptake of MTX and the response of these same tumors to a standard therapeutic regimen. While the data reported did not provide a kinetic basis for the differences in uptake observed, nor information on the steady-state concentration of MTX in these tumors, the results were highly suggestive of a role for membrane transport as a determinant of response among these tumors. The same correlation applied to cell lines with natural or acquired resistance to MTX.

Observations similar to those reported for murine tumors (16) were also made for a group of human leukemias by this same group of workers (17). Responders in both the acute lymphocytic leukemia group and acute myelocytic leukemia group were generally associated with higher rates of MTX uptake in vitro than were nonresponders. This was an extremely important finding, since it supported the notion that similar evidence derived in murine tumor models has some relevance to human cancer.

Recently, we reported on coordinated in vitro (14,18) and in vivo (5,8,9) studies that extended the earlier observations of Kessel et al (16), and correlated specific differences in kinetic parameters for transport of MTX and related folate analogs by a group of lymphoidal and nonlymphoidal murine tumors with their relative responsiveness to this agent. Similar differences were found in the transport of these antifolates by responsive murine tumors and proliferative tissue in small intestine, the drug toxicity limiting organ. These findings explain the selective antitumor action of these agents in mice.

Our initial observations in vivo (5) were made during the course of tissue pharmacokinetic experiments with the L1210 leukemia, a murine tumor highly responsive to MTX. In these studies, we documented (fig 1) a markedly greater persistence of levels of unbound intracellular MTX in tumor cells as compared to small intestine following administration of a therapeutic dose. The difference in persistence correlated with a greater duration of inhibition

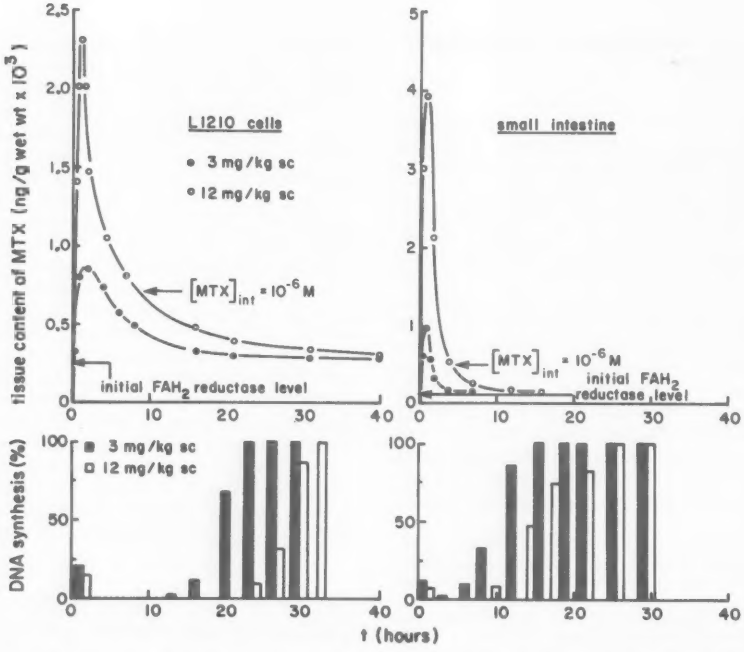

FIGURE 1.—Accumulation and retention of MTX in vivo in L1210 cells and intracellular compartment of mouse small intestine and the relationship to effects on DNA synthesis. Average values derived for two to four animals in three replicate experiments (SD $< \pm 30\%$). *See text for further details.* FAH_2=dihydrofolate.

of [^{3}H] UdR incorporation into DNA of the tumor cells. There was a similar accumulation of drug initially and a similar time course for onset of inhibition of [^{3}H] UdR incorporation in each tissue until inhibition was essentially complete. As the level of excess unbound drug within intracellular water decreased and approached the expected equilibrium level for drug bound to DHFR, the incorporation of [^{3}H] UdR into DNA resumed. However, the rate of decrease in unbound drug was lower and the resumption of [^{3}H] UdR incorporation far more delayed in the tumor cells. Both the extent of persistence and the duration of inhibition of [^{3}H] UdR incorporation in L1210 cells at different doses (5) correlated with the difference in increased life span obtained in each case. Although the dosage relationships were different, a similar correlation had already been shown (3,4) between lethal toxicity and drug persistence and duration of inhibition of [^{3}H] UdR incorporation in mouse small intestine. From these studies, we tentatively concluded the following: (1) greater persistence of MTX and longer duration of inhibition of DNA synthesis in tumor cells compared to small intestine appears to account for the selective antitumor action observed in this tumor model; (2) the pharmacokinetics for unbound drug in each tissue are determined by the membrane transport system in each case; (3) the transport system sustains unbound drug levels in the face of rapidly falling plasma levels; and (4) differences in the persistence of unbound drug in tumor versus small intestine represents a difference in a kinetic property of each transport system.

Our in vivo studies were extended (8,9) to include a number of other murine tumor models differing in responsiveness to antifolates. We confirmed our initial observation by showing the same differences in the persistence of unbound MTX and the duration of inhibition of DNA synthesis between small intestine and other responsive murine tumors. We also showed a similar correlation between these two parameters in each tumor and between these parameters and the responsiveness of these tumors during therapy. The pharmacokinetics for unbound drug in these tumors and small intestine are shown in figure 2 for a subcutaneously administered dose of 3 mg/kg. The half time for the net loss of unbound drug was proportional to the increase in survival time obtained when this dosage was employed against tumor-bearing mice on a standard regimen. In another series of experiments, the same dosage of MTX was given ip and the net loss of unbound drug with time compared to the time-course for onset and duration of inhibition of [^{3}H] UdR incorporation was also measured in each tissue. Again, the half time for net loss of unbound drug is proportional (fig 3) to the increase in survival time obtained during therapy in vivo. Also, the time required for intracellular exchangeable levels of drug to approach the binding equivalence for DHFR, without exception, correlates (fig 4) with the time required for the resumption of [^{3}H] UdR incorporation. Only small differences in the DHFR content were found (8,14) among these and other (19) tumor cells, and these differences do not correlate (8,14,19) with the relative responsiveness of these tumors.

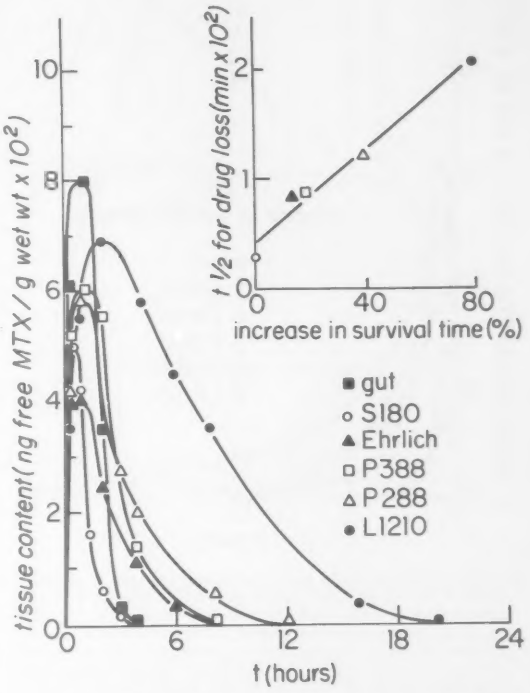

FIGURE 2.—Time course for accumulation and retention of unbound MTX in vivo in different murine tumor cells and intracellular compartment of mouse small intestine following a single subcutaneous dose of 3 mg/kg. The inset shows the relationship between the half time for drug loss and the increase in survival time. Average values derived for two to four mice in two separate experiments (SD < ±30%). Data on average survival times were obtained in two separate experiments with 10 mice in each control and treated group (SD < ±15%). *See text for further details.* For reasons of brevity, the term gut has been used in the figure to designate small intestine.

In other studies (8,9,18), we associated differences in lethal potency of 10-ethylaminopterin, MTX, aminopterin (relative potency = 16:14:1), methasquin, and 5-Cl-5-deaza-aminopterin (relative potency = 5:0.3) with differences (fig 5) in the persistence of unbound levels of each in the intracellular compartment of small intestine. After a dose of 3 mg/kg given sc or ip, persistence was in the order 5-Cl-deaza-aminopterin > aminopterin > methasquin > MTX ≃ 10-ethylaminopterin. A similar correlation was derived (8,9) (*see also* fig 6) for these agents when given at equitoxic doses. Moreover, differences in the therapeutic index for these five agents against the L1210 leukemia (8,9,18) (*see also* fig 6) was related to the

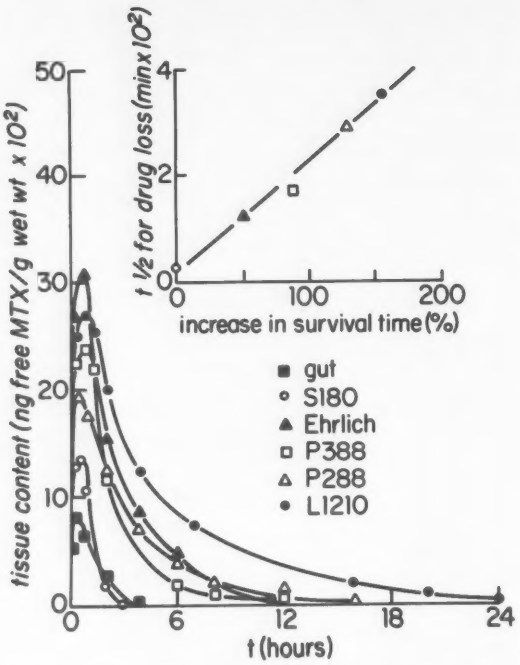

FIGURE 3.—Time course for accumulation and retention of unbound MTX in vivo in different murine tumor cells and intracellular compartment of mouse small intestine following a single *intraperitoneal* dose of 3 mg/kg. The inset shows the relationship between the half time for drug loss and the increase in survival time. Average values derived for two to four mice in two separate experiments (SD<±30%). Data on average survival times were obtained in two separate experiments with 10 mice in each control and treated group (SD<±18%). Data shown in figures 3 and 4 were obtained in the same experiment. *See text for further details.* For reasons of brevity, the term gut has been used in the figure to designate small intestine.

persistence of each agent at maximum tolerated doses and also to the duration of inhibition of [^{3}H] UdR incorporation into DNA in tumor cells vis a vis small intestine. These studies explain, at least in mice, the greater lethal potency of aminopterin, methasquin, and 5-chloro-5-deaza-aminopterin and the higher therapeutic index observed for MTX and 10-ethylaminopterin.

In more recent in vivo studies (20), we examined the pharmacokinetics for MTX in mouse bone marrow in an attempt to explain the dose limiting toxicity of the small intestine in this species (21). Following sc administration of varying doses of MTX (12 to 400 mg/kg) to mice, accumulation and persistence of drug in marrow was markedly less (fig 7) in comparison to that seen in small intestine. In addition, the lower persistence of unbound drug in marrow versus small intestine correlated with a substantially shorter duration of inhibition of DNA synthesis in the former. Earlier recovery of DNA synthesis as a consequence of more rapid loss of unbound drug appears to explain the large differences in drug sensitivity of these two tissues. These results and those of prior studies from our laboratory (5,7–9) and elsewhere (6) are consistent with the observed differences in the cytotoxicity of MTX in various tissues in the order tumor >> small intestine > marrow for responsive tumor models such as the L1210, P288, or P388 leukemias.

TISSUE-SPECIFIC DIFFERENCES IN MEMBRANE TRANSPORT

During more recent studies in vitro (14,18) we were able to provide direct evidence, which confirmed the predictions derived from our pharmacokinetic data (5,8,9), relating the role of membrane transport to both the relative responsiveness among tumors and the selective antitumor action obtained with antifolates. We were able to identify the K_m for influx of methotrexate, rather than the V_{max} for influx or the efflux rate constant, as the parameter of the transport system for antifolates that had the highest correlation (14) with relative therapeutic responsiveness. Both the relatively unresponsive tumors (Sarcoma 180, Ehrlich carcinoma) and the responsive tumors (L1210, P288, and P388 leukemias) appear (14) to possess transport systems for MTX with similar properties. Relative responsiveness among this group of tumors as determined from the increased life span (%ILS) in vivo following treatment with MTX shows (fig 8) a linear relationship with the steady-state level of unbound drug over a fourfold range. Although some differences were observed (14) in the individual values for V_{max}, these were relatively small (<50%), and might merely reflect differences in cell size. These small differences in V_{max} alone showed no correlation with relative responsiveness among these tumors. The relationship between steady-state levels of unbound drug and relative responsiveness, with one exception (P388), could be explained entirely by a difference in saturability of influx among these tumors (variation in K_m=3.13=11.3 μM). The single exception could be explained by a difference (14) in the value for the efflux rate constant. Although the differences in responsiveness observed in this study were explained largely by the differences in the value for the influx K_m, it should be emphasized that it is not these differences per se that account for relative responsiveness, but the net effect of these differences on the accumulation of free drug in each tumor. The quantitative relationship between the values of spe-

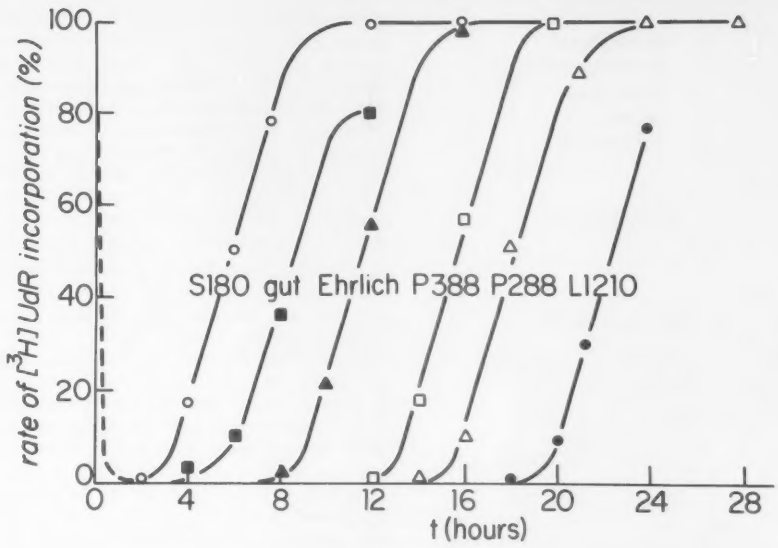

FIGURE 4.—The incorporation of [^{3}H] UdR into DNA in vivo in mouse small intestine and in different tumor cells at various times after a single *intraperitoneal* dose of 3 mg/kg MTX. Data are expressed as percentage of control rates of incorporation in two to four mice in two separate experiments (SD$<\pm$30%). Data shown in figures 3 and 4 were obtained in the same experiment. For reasons of brevity, the term gut has been used in the figure to designate small intestine.

cific kinetic parameters for transport (K_m or steady-state) and relative responsiveness shown for the poorly responsive Sarcoma 180 and Ehrlich tumors approximates the same relationship shown (22 and fig 8) or inferred (16) for L1210 cell lines with acquired resistance to MTX.

The role of membrane transport in selective antitumor action of folate analogs in murine tumor models was also addressed via a series of in vitro experiments reported very recently (18) from our laboratory. Epithelial cell populations were isolated from mouse small intestine, the site of drug-limiting toxicity in these animals. These suspensions, consisting of approximately equal numbers of immature crypt and mature columnar epithelial cells (18), rapidly accumulated folate analogs by carrier-mediation in a manner similar to that observed (14) for tumor cells. The kinetic analysis of influx delineated only a single saturable component with a value for apparent K_m of approximately 90 μM. Some difference between isolated epithelial cells and L1210 cells (*see* table 1) was observed in all the kinetic parameters examined. The V_{max} (twofold) and the efflux rate constant (threefold) were lower in the epithelial cell suspensions. However, the influx K_m for epithelial cells was approximately 25-fold higher than the value derived with L1210 cells. The same kinetic data were also derived (18 and table 1) for two other analogs, aminopterin and 10-ethylaminopterin, that compete with MTX. Values for the efflux rate constant and the influx V_{max} in each tissue were similar for all three analogs. In each cell type, the alkyl substitution resulted in a decrease in the value for influx K_m. However, the decrease was fourfold greater in the case of epithelial cells. The relative cytotoxic consequences anticipated from these kinetic differences can be derived from calculated values for steady-state, which are shown for a concentration of 1 μM in table 1. At this concentration, values derived for steady-state levels of exchangeable drug in L1210 cells versus epithelial cells are only twofold higher for aminopterin, but 12-fold higher for the two alkyl substituted analogs. The correlation between the individual kinetic values and calculated values for steady-state level shown in table 1 and the actual pharmacokinetic data derived from earlier (8,9,18 and figs 1 through 6) and companion studies in vivo is striking. The results of these in vitro studies would appear to substantiate the role of transport with regard to the selective antitumor action of this class of compound in murine tumor models. They also identify a difference in specificity of transport systems of tumor and epithelial cells of small intestine for N^{10}-substituents as the molecular basis for the differences observed between cell types.

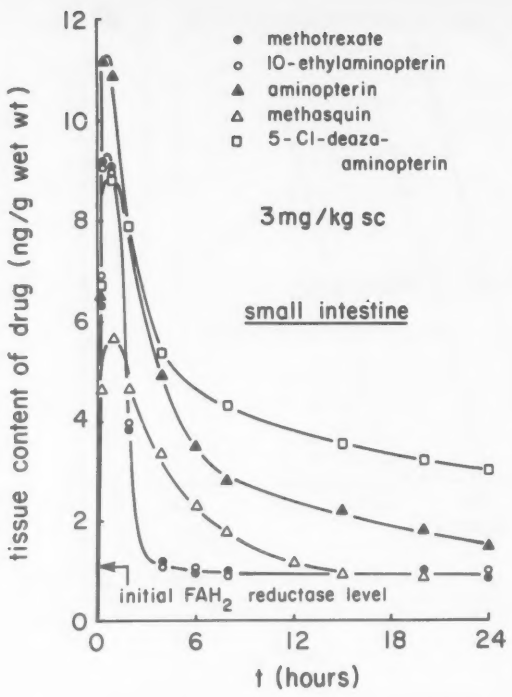

FIGURE 5.—Time course for accumulation and retention of different folate analogs in vivo in mouse small intestine. The analogs were administered subcutaneously as a single dose of 3 mg/kg. Average values were derived for drug content of the intracellular compartment of small intestine from three to four animals in three replicate experiments. (SD<±30%). *See text for further details.* FAH_2=dihydrofolate.

CONCLUSIONS

The overall significance of these findings to folate analog pharmacology is several-fold. They establish the same pharmacokinetic basis for differences in recovery of normal proliferative tissues as well as various tumor tissues following antifolate therapy. Moreover, the degree of documentation provided in these studies now allows us to propose, with some measure of confidence, that the property for accumulating and maintaining pharmacologically effective intracellular levels of folate analogs is different amongst all proliferative tissues of this rodent and, probably, in all mammals. These findings also delineate a concept for the basis of selective drug action that takes into consideration antiproliferative effects against tumor within the context of drug-limiting effects on normal host tissues. In so doing, further validity is derived for an extrapolation of a concept elucidated in animal models to patients where antifolate effects in bone marrow are as serious as in gastrointestinal tissue.

Although the relative responsiveness and selective antitumor action observed during folate analog therapy of these murine tumor models appear to be explained largely within the framework of current knowledge of carrier-mediated membrane transport, we would only tentatively suggest that there may be a similar basis for related observations in other tumor models or in patients. Certainly, the possibility must be considered that factors other than those related to membrane transport per se may play a contributing, if not a determining role, in specific cases. For instance, differences in the extent of methotrexate inhibition have been reported (23) for DHFR isolated from different rodent and human tumor cell lines, which shows some correlation with growth inhibition of these cell lines in vitro. However, it will be necessary to provide some documentation for the existence of these differences in situ and evidence for their therapeutic significance at a pharmacologic level. Some correlation has also been shown (24) between the relative oxidation rates of folate analogs to the 7-OH derivative by liver aldehyde oxidase and toxicity in different animal species. Any relevance to the difference in toxicity of these two agents in rodents and man, however, is doubtful, since the overall level of activity of these enzymes is so low. Lastly, enzyme mediated polyglutamation may enhance the toxicity of MTX, or other folate analogs, in some tissues more than others because of greater retention and/or inhibitory effects of these derivatives on other folate utilizing enzymes. In regard to the former, our own studies of the γ-glutamyl peptide of MTX suggest (15) a rate of loss of this peptide derivative from L1210 cells equal to MTX itself. Although the metabolism of folate analogs of the sort described above is well documented for a variety of mammalian tissues, no evidence has been reported so far for a direct involvement in the selective antitumor action of these agents in animal models or patients.

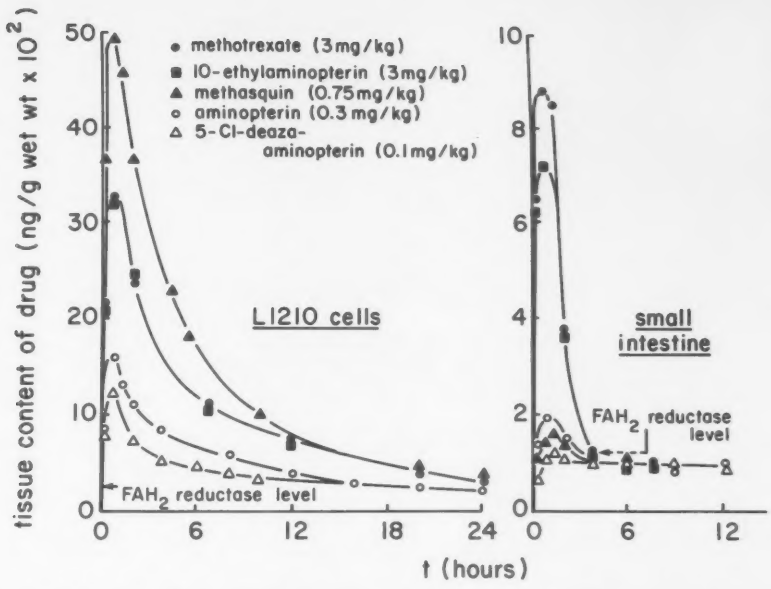

FIGURE 6.—Time course for accumulation and retention of different folate analogs in vivo in L1210 leukemia cells and mouse small intestine. The analogs were administered subcutaneously as a single dose, which was shown to be equitoxic (LD_{10}) on a therapeutic schedule of once every 2 weeks × 10. Average values were derived for drug content of tumor cells and the intracellular compartment of small intestine from three to four animals in three replicate experiments (SD < ±30%). *See text for further details.* FAH_2=dihydrofolate.

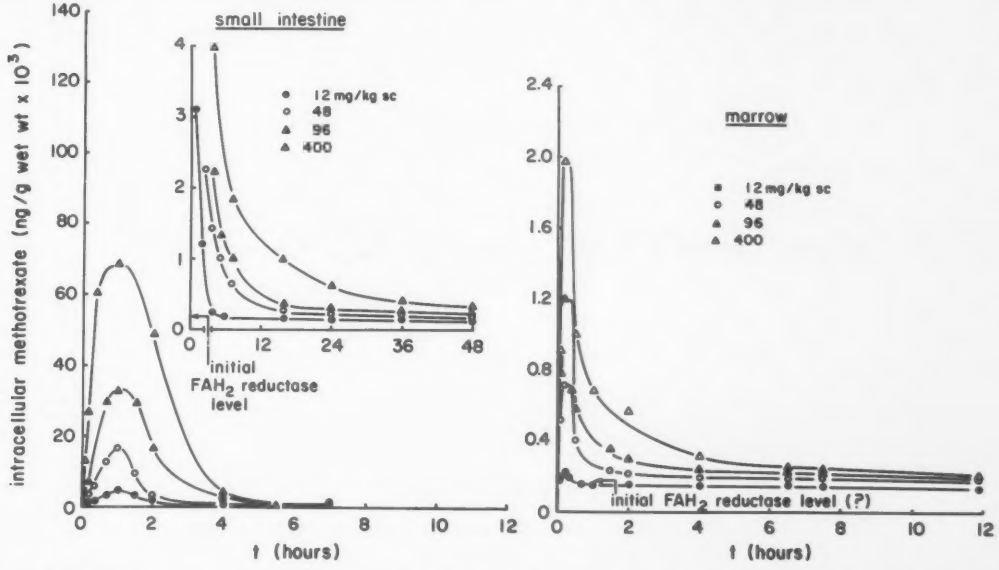

FIGURE 7.—MTX pharmacokinetics in bone marrow and small intestine in non-tumor-bearing mice. Drug was administered subcutaneously. Values shown were derived in triplicate experiments done on different days employing two or three mice for each determination. Standard deviation did not exceed ±26% of mean values. FAH_2=dihydrofolate.

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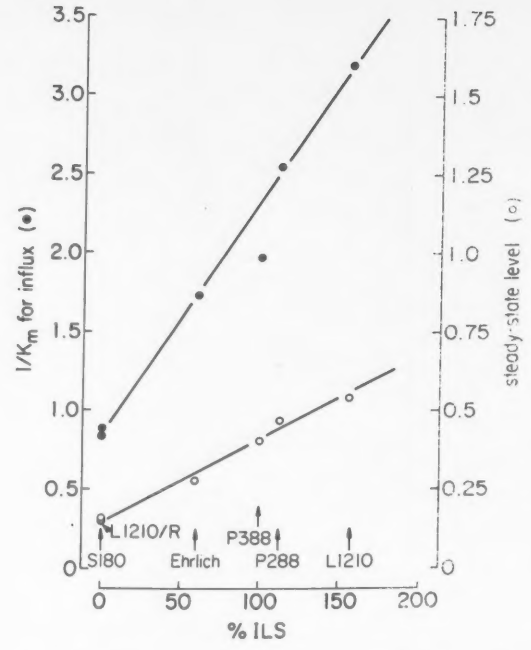

FIGURE 8.—The relationship between parameters for MTX transport in vitro and relative therapeutic responsiveness of murine tumors in vivo. K_m, μM; steady-state, μmol/g dry wt $[MTX]_{ext} = 0.44$ μM; ILS, increased life span obtained when tumor-bearing mice were treated every other day with 3 mg/kg ip MTX (three separate experiments, SD = < ±24%).

REFERENCES

1. WERKHEISER WC. The biochemical, cellular and pharmacological action and effects of the folic acid antagonists. Cancer Res 23:1277–1285, 1963.
2. BERTINO JR. The mechanism of action of folate antagonists in man. Cancer Res 23:1286–1306, 1963.
3. MARGOLIS S, PHILIPS FS, and STERNBERG SS. The cytotoxicity of methotrexate in mouse small intestine in relation to inhibition of folic acid reductase and of DNA synthesis. Cancer Res 31:2037–2046, 1971.
4. PHILIPS FS, SIROTNAK, FM, SODERGREN JE, ET AL. Uptake of methotrexate, aminopterin and methasquin and inhibition of dihydrofolate reductase and of DNA synthesis in mouse small intestine. Cancer Res 33:153–158, 1973.
5. SIROTNAK FM, and DONSBACH RC. Differential cell permeability and the basis for selective activity of methotrexate during therapy of the L1210 leukemia. Cancer Res 33:1290–1294, 1973.
6. CHABNER BA, and YOUNG RC. Threshold methotrexate concentration for in vivo inhibition of DNA synthesis in normal and tumorous target tissues. Clin Invest 52:1804–1811, 1973.
7. SIROTNAK FM, and DONSBACH RC. The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. Cancer Res 34:3332–3340. 1974.
8. SIROTNAK FM, and DONSBACH RC. Further evidence for a basis of selective activity and relative responsiveness during antifolate therapy of murine tumors. Cancer Res 35:1737–1744, 1975.
9. SIROTNAK FM, and DONSBACH RC. A basis for the difference in toxicity of methotrexate, aminopterin and methasquin in mice. Biochem Pharmacol 24:156–158, 1975.
10. GOLDMAN ID, and FYFE MJ. The mechanism of action of methotrexate. 1. Interaction with a low affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell fibroblasts. Mol Pharmacol 10:275–282, 1974.
11. WHITE JC, and GOLDMAN ID. Mechanism of action of methotrexate. IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells in vitro. Mol Pharmacol 12:711–719, 1976.
12. JACKSON RC, and HARRAP KR. Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.
13. GOLDMAN ID. Analysis of the cytotoxic determinants for methotrexate (NSC-740): a role for "free" intracellular drug. Cancer Chemother Rep 6:51–62, 1975.

TABLE 1.—Values for individual kinetic parameters of folate analog transport by L1210 cells and by isolated murine intestinal epithelial cells.

Cell type	Analog	Influx		Efflux	Calculated steady state	
		K_m (μM)	V_{max} (nmol/min/g dry wt)	K† (min $^{-1}$)	(nmol/g dry wt)	(μM)§
L1210	aminopterin	1.22±0.10*	3.27±1.1	0.25±0.03	5.94	1.73
	MTX	3.52±0.53	4.55±1.4	0.25±0.07	4.01	1.21
	10-ethylaminopterin	3.30±0.83	4.32±1.5	0.23±0.02	4.29	1.18
intestinal epithelium	aminopterin	7.23±3.64	1.74±0.48	0.084±0.01	2.52	0.83
	MTX	87.06±6.34	2.41±0.31	0.077±0.02	0.34	0.11
	10-ethylaminopterin	123.77±17.90	2.56±0.5	0.067±0.01	0.31	0.10

*Average value±SD (n = 3 to 5 separate determinations on different days).
†$K = \text{Ln } 2/t_{½}$

$$C_{\infty} = \frac{V_{max}[drug]}{(K_m + [drug])\,K_e} \quad [drug] \simeq 1\,\mu M$$

§Calculated from values of intracellular water/g dry wt of 3.67 ml ±0.54 (L1210 cells) and 2.98 ±0.3 (epithelial cells). *See text for related methodology.*

14. Sirotnak FM, and Donsbach RC. Kinetic correlates of methotrexate transport and therapeutic responsiveness in murine tumors. Cancer Res 36:1151–1158, 1976.
15. Sirotnak FM, Chello PL, Piper JR, et al. Growth inhibitory, transport and biochemical properties of the γ-glutamyl and γ-aspartyl peptides of methotrexate in L1210 leukemia cells in vitro. Biochem Pharmacol 27:1821–1825, 1978.
16. Kessel D, Hall TC, and Roberts D, et al. Uptake as a determinant of methotrexate response in mouse leukemias. Science 150:752–754, 1965.
17. Kessel D, Hall TC, Roberts D. Modes of uptake of methotrexate by normal and leukemic lymphocytes in vitro and their relationship to drug response. Cancer Res 28:564–570, 1968.
18. Chello PL, Sirotnak FM, Dorick DM, et al. Therapeutic relevance of differences in the structural specificity of the transport systems for folate analogs in L1210 tumor cells and in isolated murine intestinal epithelial cells. Cancer Res 37:4297–4303, 1977.
19. Roberts D, Wodinsky I, and Hall TC. Studies on folic reductase. III. The level of enzyme activity and response to methotrexate of transplantable mouse tumors. Cancer Res 25:1899–1903, 1965.
20. Sirotnak FM, and Moccio DM. Pharmacokinetic basis for differences in methotrexate sensitivity of normal proliferative tissues in the mouse. Cancer Res. 1979. In press.
21. Ferguson FC (jr), Thiersch JB, and Philips FS. The action of 4-amino-N^{10}-methyl-pteroylglutamic acid in mice, rats and dogs. J Pharmacol Exp Ther 98:293–301, 1950.
22. Sirotnak FM, Kurita S, and Hutchison DJ. On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. Cancer Res 28:75–80, 1968.
23. Jackson RC, Hart LI, and Harrap KR. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductase. Cancer Res 36:1991–1997, 1976.
24. Johns DJ, Ionatti AT, Sartorelli AC, et al. The relative toxicities of methotrexate and aminopterin. Biochem Pharmacol 15:555–561, 1965.
25. Whitehead VM. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. Cancer Res 37:408–412, 1977.

The Role of Methotrexate and Dihydrofolate Polyglutamates in the Enhancement of Fluorouracil Action by Methotrexate [1]

D. J. Fernandes,[2,3,*] B. A. Moroson,[3] and J. R. Bertino [4]

SUMMARY

The effect of the methotrexate (MTX)-5-fluorouracil (FUra) combination on L1210 cell viability was highly sequence dependent in that a 1.4 log (25-fold) increase in cytotoxicity was observed when the MTX exposure preceded FUra, as compared to the reverse sequence.

The formation of ternary complexes of thymidylate synthetase has been examined as a basis for the interaction of FUra and MTX in L1210 cells. L1210 cells converted 39% of the total intracellular MTX into MTX-poly-γ-glutamates within 4 hours of a 1 μM MTX exposure. MTX-diglutamate (2,4-diamino,N [10]-methylpteroyldiglutamate) and MTX-triglutamate were the predominant metabolites. In contrast to the ternary complexes formed with MTX and MTX-diglutamate, the 5-fluorodeoxy-uridylate (FdUMP)-7,8-dihydropteroylpentaglutamate-enzyme and the FdUMP-5,10-methylenetetrahydropteroylpentaglutamate-enzyme complexes were stable to polyacrylamide gel electrophoresis under nondenaturing conditions. MTX-diglutamate enhanced the extent of tight-binding inhibition of thymidylate synthetase activity by FdUMP in the presence of saturating 5,10-methylenetetrahydropteroylpentaglutamate, suggesting that MTX-diglutamate did not antagonize the formation of FdUMP-5,10-methylenetetrahydropteroylpentaglutamate enzyme complex. We propose that the sequence-dependent effect of MTX plus FUra on L1210 cell viability results from MTX and MTX polyglutamate inhibition of dihydrofolate reductase, and consequently a trapping of intracellular folates as dihydrofolate polyglutamates that could increase the extent of FdUMP binding to thymidylate synthetase.

[Cancer Treat Rep 65 (Suppl. 1):29–35, 1981]

Recent studies of combination chemotherapy of murine tumor cells propagated in vitro (1,2) as well as in vivo (3–5), have demonstrated a sequence-dependent cell kill effect of methotrexate (MTX) and 5-fluorouracil (FUra). Thus, enhanced cell kill was noted with the sequence MTX followed by FUra, whereas the opposite schedule gave less than additive effects. Enhanced toxicity to normal tissues was not observed with the MTX → FUra sequence (3). We hypothesized that MTX pretreatment followed by FUra was synergistic by virtue of enhanced binding of FdUMP, an active metabolite of FUra, to thymidylate synthetase in the presence of the folate analog (3).

In this communication we describe studies of complex formation among thymidylate synthetase purified from L1210 cells, FdUMP, and MTX. Since folates (6) and MTX (7) have been shown to exist in cells predominantly as poly-γ-glutamyl conjugates, we have also examined the formation of ternary complexes of folate and MTX polyglutamates with FdUMP and thymidylate synthetase.

MATERIALS AND METHODS

MTX [5] (Na salt) was obtained from the Division of Cancer Treatment, NCI. [3′,5′,7-^{3}H]MTX (Na salt) was

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[5] Abbreviations used: MTX, methotrexate, 2,4-diamino-N[10]-methylpteroyl-glutamate; FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxy-

Continued

purchased from the Amersham Corporation and purified on DEAE cellulose. MTX (Glu_1) was the generous gift of Dr. Samuel Jacobs, University of Pittsburgh School of Medicine. MTX (Glu_2) and MTX (Glu_3) were kindly provided by Dr. Charles Baugh, University of Alabama School of Medicine. FUra was obtained from Hoffmann-LaRoche, Inc.; dUMP and FdUMP (Na salts), Triton X-100, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. dUMP-[5-^{3}H] was purchased from Amersham Corp. FdUMP-[6-^{3}H] was obtained from Moravek Biochemicals. (d,ℓ)-L-H_4PteGlu was a gift of Dr. John McGuire, Department of Pharmacology, Yale University School of Medicine. 7,8-H_2PteGlu$_5$ was kindly provided by Dr. James Coward and A. R. Cashmore, Department of Pharmacology, Yale University, and was converted to (ℓ)-L-H_4PteGlu$_5$ by reaction with dihydrofolate reductase (8). No contamination of H_2PteGlu$_5$ with either H_4PteGlu$_5$ or 5,10-CH_2-H_4PteGlu$_5$ was detected either spectrally or by reversed phase high performance liquid chromatography. Biogel P-100 (100 to 200 mesh) was purchased from Bio-Rad Laboratories. Fischer's medium and horse serum were obtained from Grand Island Biological Co. The composition of buffers was as follows: Buffer A, 50 mM Tris-HCl pH 7.5, 10% glycerol (v/v), and 2 mM DTT; buffer B, 250 mM Tris-HCl, pH 7.5, 10% glycerol, 13 mM 2-mercaptoethanol and 6 mM formaldehyde; buffer C, 50 mM Tris-HCl pH 7.5, 10% glycerol, 20 mM 2-mercaptoethanol, 3 mM formaldehyde and 42 mM NaCl; buffer D, 50 mM Tris-HCl pH 7.5, 10% glycerol, 20 mM 2-mercaptoethanol, and 168 mM NaCl. The concentrations of all drugs and folates were determined spectrophotometrically.

Soft Agar Cloning

The viability of L1210 cells after drug exposure was assayed by cloning in soft agar as described by Chu and Fischer (9).

Enzyme Purification

L1210 thymidylate synthetase was purified by N^{10}-formyl-5,8-dideazafolate affinity chromatography according to the method of Rode et al (10). L1210 leukemia cells propagated ip in CDF_1 mice were used as a cell source. The electrophoretically homogeneous preparation had a specific activity of 383 nmol/minute/mg of protein. Protein concentrations were estimated by the Coomasie blue staining method (11).

Enzyme Assays

Thymidylate synthetase was assayed by the tritium displacement method of Roberts (12) as modified by Dolnick and Cheng (13). Tritium release was linear with respect to time and protein concentration under the conditions of all experiments. Once enzyme unit is defined as the quantity of enzyme required to form 1 nmol of dTMP/minute at 37 °C under our assay conditions, enzyme concentrations were calculated by titration of thymidylate synthetase with FdUMP in the presence of saturating 5,10-CH_2-H_4PteGlu.

Biogel P-10 Chromatography

L1210 thymidylate synthetase at a concentration of 10 nM was incubated in buffer B for 10 minutes at 37 °C with 8.5 nM [^{3}H]-5-FdUMP (11.1 Ci/mmol) in combination with either 40 μM MTX, 10 μM MTX(Glu_1), or 410 μM 5,10-CH_2-H_4PteGlu. Enzyme activity was stable and binding of FdUMP was maximal during this 10-minute incubation. The reaction mixtures were placed on a 0.9 × 30 cm column (bed volume of 6.0 ml) that had been equilibrated with buffer A. Elution was carried out at 4 °C with buffer A at a flow rate of 0.9 ml/minute. Radioactivity was counted in 10 ml of ACS scintillation fluid (Amersham) in a Beckman LS-230 liquid scintillation spectrometer until a statistical error of ± 5 % was reached.

Quantitation of Intracellular MTX and MTX Polyglutamates

L1210 leukemia cells were grown in Fischer's media supplemented with 10% (v/v) horse serum. Cells in the logarithmic phase of growth at a density of 1 × 10^5 cells/ml were incubated for 4 hours with 1 μM [^{3}H]MTX at a final specific radioactivity of 0.73 Ci/mmol in a volume of 25 ml. At the end of the incubation the cells were washed twice with drug-free media at 4 °C and the [^{3}H] extracted by boiling for 10 minutes in 0.1 M Na-phosphate pH 5.5. The extract was centrifuged at 27,000 × g for 30 minutes at 4 °C, and the supernatant was analyzed by high performance liquid chromatography as previously described (14). [^{3}H]MTX polyglutamates were identified by their retention times relative to authentic standards.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed as described by Davis (15) with no stacking gel. The gels were frozen on solid CO_2, cut into 1.5 mm sections, and solubilized by heating at 50 °C in H_2O_2-NH_4OH (11:1 v/v). Radioactivity was counted as described above.

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uridylate; MTX(Glu_1), methotrexate-diglutamate; MTX(Glu_2) MTX-triglutamate, etc.; H_4PteGlu, 7,8-dihydropteroylglutamate, 7,8-dihydrofolate; H_4PteGlu$_5$, 7,8-dihydropteroylpentaglutamate; 5,10-CH_2-H_4PteGlu, (d, ℓ)-5,10/methylene-5,6,7,8-tetrahydropteroylglutamate, racemic mixture of the physiologically active diastereoisomer (ℓ) and the nonphysiological diastereoisomer (d), 5,10-methylenetetrahydrofolate; 5,10-CH_2-H_4PteGlu$_5$. (ℓ)-5,10-methylene-5,6,7,8-tetrahydropteroylpentaglutamate.

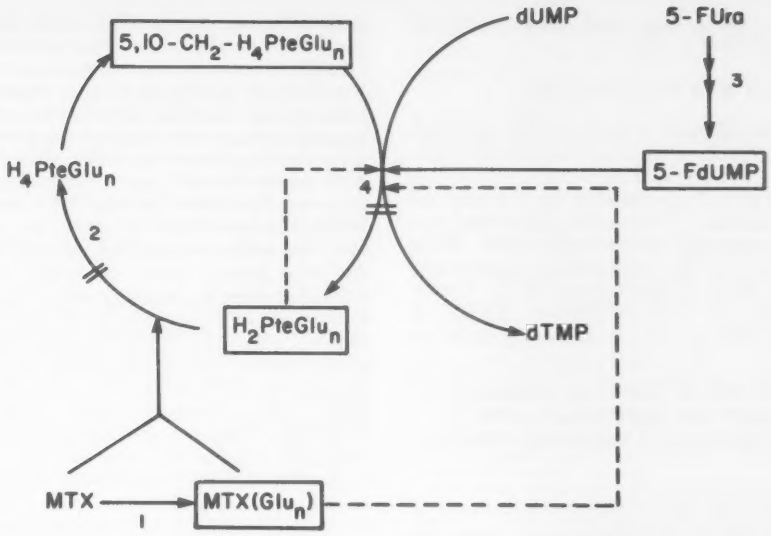

FIGURE 1.—Relationship among MTX, 5-FUra, and thymidylate synthetase. MTX is converted by folylpolyglutamate synthetase (1) to MTX poly-γ-glutamates. Both MTX and MTX(Glu_n), by inhibiting dihydrofolate reductase (2) block the generation of tetrahydropteroylpolyglutamates, which results in the accumulation of dihydropteroylpolyglutamates. 5-FUra is anabolized along the pathways available for uracil to 5-FdUMP (3) which in the presence of 5,10-$CH_2H_4PteGlu_n$ is a tight binding inhibitor of thymidylate synthetase (4) MTX(Glu_n) and $H_2PteGlu_n$ may also form ternary complexes with 5-FdUMP and thymidylate synthetase.

RESULTS

L1210 cells, propagated in vitro, were selected as a model system after confirming that the effect of combinations of MTX and FUra on cell viability was highly dependent on the sequence of drug administration (table 1). A 4-hour exposure to 10 μM FUra followed by a 4-hour exposure of 1 μM MTX was antagonistic in inhibiting soft-agar colony formation, since the 3.7 log reduction in cell number was less than the sum of the log cell kill of 4.5 obtained with FUra (1.7 log kill) plus MTX (2.8 log kill). Conversely, when cells were exposed to MTX prior to FUra, a 5.1 log reduction in tumor cell viability was seen, which was greater than the sum of the FUra and MTX effects. Simultaneous FUra plus MTX produced an additive response. This study was repeated twice with similar results.

The proposed relationship among FUra and MTX metabolism, and thymidylate synthetase ternary complex formation is illustrated in figure 1. We postulated that the sequence-dependent effect of FUra and MTX on cell viability was related to inhibition of thymidylate synthetase via formation of ternary enzyme complexes. It was reasoned that exposure of L1210 cells to MTX before FUra produced a synergistic cell kill because it resulted in either (a) the synthesis and accumulation of MTX polyglutamates prior to the FUra exposure, which subsequently could stimulate tight binding of FdUMP to thymidylate synthetase; or (b) inhibition of dihydrofolate reductase and consequently an increase in dihydrofolate

TABLE 1.—Sequence dependent effects of MTX and FUra on L1210 cell viability

Condition	Log cell kill
FUra	1.7±0.05*
MTX	2.8±0.34
FU→MTX	3.7±0.14
MTX+FUra	4.7±0.24
MTX→FUra	5.1±0.25

*Mean ±SD(N=4)

L1210 cells grown at a density of 2×10^5 cells/ml were exposed to either 1 μM MTX or 10 μM FUra. Each drug exposure either alone or in combination was for 4 hours. Cells were washed once in drug-free media after each 4-hour exposure and cloned in soft agar. Cell viability was determined as described in METHODS. Cloning efficiency of untreated cells was 89%.

polyglutamates, which also could enhance FdUMP binding to thymidylate synthetase.

Biosynthesis of MTX Polyglutamates

Initial experiments were directed toward determining if MTX polyglutamates were formed intracellularly during the 4-hour exposure to MTX. L1210 cells were incubated with 1 μM [^{3}H]MTX for 4 hours, and the cells were washed and extracted as described elsewhere. Chromatography on reversed phase HPLC showed that 39% of the intracellular radioactivity was present as MTX polyglutamates (fig 2). The intracellular levels of MTX, MTX(Glu_1), MTX(Glu_2), and MTX(Glu_3) were 2, 4, 0.4, and 1.0 μM, respectively.

Effects of MTX and MTX(Glu_1) on Binding of FdUMP to Thymidylate Synthetase in the Presence and Absence of Saturating 5,10-CH_2-H_4PteGlu

The effect of MTX and MTX(Glu_1) in enhancing binding of FdUMP to thymidylate synthetase was studied by reacting purified L1210 thymidylate synthetase (10 nM) with a limiting concentration of [^{3}H]-FdUMP (8.5 nM) in either the presence or absence of the folate or antifolate cofactor (table 2). The total number of 5,10-CH_2-H_4PteGlu-dependent FdUMP binding sites on thymidylate synthetase was determined by titration of thymidylate synthetase activity with excess FdUMP and 5,10-CH_2-H_4PteGlu. In the absence of cofactor, a negligible amount of [^{3}H]-FdUMP coeluted with thymidylate synthetase activity in the void volume of the column. MTX at 40 μM and to a greater extent MTX(Glu_1) at 10 μM were somewhat effective in stimulating binding of FdUMP to thymidylate synthetase. In the presence of saturating (410 μM) 5,10-CH_2-H_4PteGlu, 8.5 nM FdUMP was able to titrate 35% of the FdUMP binding sites. The addition of MTX did not increase FdUMP binding over that obtained with 5,10-CH_2-H_4PteGlu. MTX(Glu_1) enhanced the extent of ternary complex formation even in the presence of a saturating concentration of 5,10-CH_2-H_4PteGlu. Binding of FdUMP in the presence of MTX(Glu_1) plus 5,10-CH_2-H_4PteGlu (60% titration of FdUMP binding sites) was approxi-

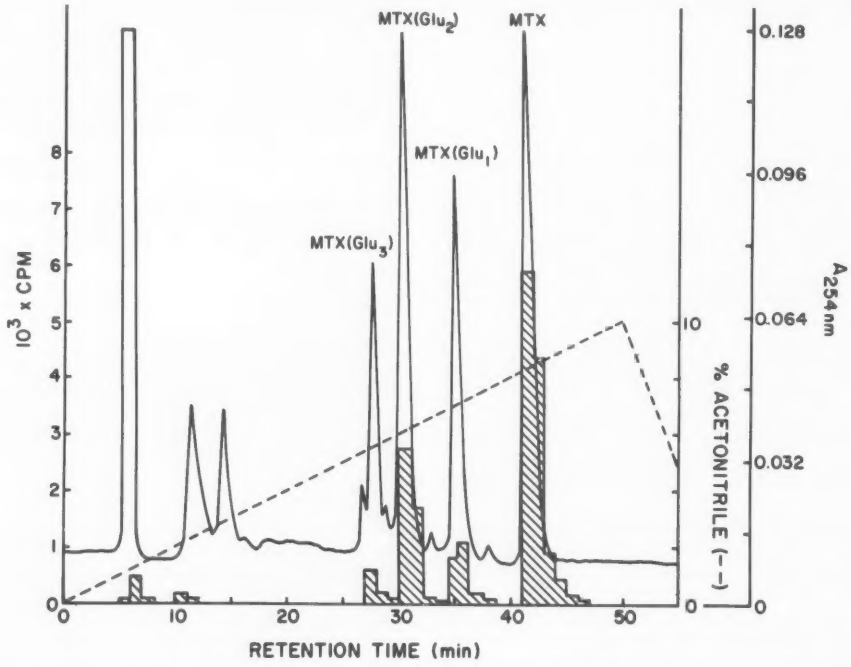

FIGURE 2.—Reversed phase high performance liquid chromatography of extracts of L1210 cells incubated for 4 hours with 1 μM [^{3}H]MTX at a final specific radioactivity of 073 Ci/nmol. Hatched area [^{3}H]CPM; —, A_{254nm} of authentic standards MTX, MTX(Glu_1), MTX(Glu_2), and MTX(Glu_3).

mately additive compared to binding in the presence of MTX(Glu_1) (19%) and 5,10-CH_2-H_4PteGlu (35%), alone. Similar results were obtained when inhibition of thymidylate synthetase activity was quantitated as an indirect measurement of FdUMP binding (data not shown). These results appeared reasonable based on studies of L1210 (data not shown) and human (16) thymidylate synthetase, which indicated that MTX was a noncompetitive inhibitor with respect to 5,10-CH_2-H_4PteGlu, and multiple inhibitor studies, which revealed two distinct folate binding sites on human thymidylate synthetase (13).

TABLE 2.—Effect of MTX and MTX(Glu_1) on binding of FdUMP to thymidylate synthetase in the presence of saturating 5,10-CH_2-H_4PteGlu

Reactants	[^{3}H]FdUMP bound (10^3 x DPM)	[^{3}H]FdUMP bound FdUMP binding sites
E+FdUMP	1.4	<0.01
E+FdUMP+MTX	56±8*	0.13
E+FdUMP+MTX(Glu_1)	82±12	0.19
E+FdUMP+5,10-CH_2-H_4PteGlu	152±23	0.35
E+FdUMP+MTX+5,10-CH_2-H_4PteGlu	153±24	0.36
E+FdUMP+MTX(Glu_1)+5,10-CH_2-H_4PteGlu	251±35	0.60

*Mean ± SD (N = 3).

Thymidylate synthetase at a concentration of 10 nM was incubated for 10 minutes in buffer B at 37 °C with 8.5 nM [^{3}H]-FdUMP (11.1 Ci/mmol) in combination with 40 μM MTX, 10 μM MTX (Glu_1), and 410 μM 5,10-CH_2-H_4PteGlu. The reaction mixtures were placed on Biogel P-10 chromatography columns and elution was carried out at 4° C.

Effect of Reduced Folate Polyglutamates on Tight-Binding Inhibition of Thymidylate Synthetase

Since the folates in most cells contain predominantly 5 glutamyl residues (17), it appeared important to examine the effect of 5,10-CH_2-H_4PteGlu$_5$ and 7,8-H_2PteGlu$_5$ on FdUMP binding to thymidylate synthetase. The dihydro-compound was of particular interest, since its intracellular concentration is known to increase markedly after cells are exposed to MTX (18,19).

L1210 thymidylate synthetase was preincubated with inhibitors and aliquots removed and assayed for enzyme activity in a 21-fold dilution of assay mix. Under these conditions only tight-binding inhibition was measured, since weak complexes would dissociate in the assay and thereby have little effect on enzyme activity. Thus, as indicated in table 3, neither 10 μM of the natural (ℓ) isomer of 5,10-CH_2H_4PteGlu$_5$ nor 18 μM 7,8-H_2PteGlu$_5$ alone produced any statistically significant inhibition of thymidylate synthetase activity. However, preincubation of 5,10-CH_2-H_4PteGlu$_5$ (10 μM) with FdUMP resulted in nearly a complete loss of enzyme activity. 7,8-H_2-PteGlu$_5$ at 18 μM also increased the affinity of FdUMP to thymidylate synthetase. The maximal effect of 7,8-H_4-PteGlu$_5$ could not be tested with this assay, since 7,8-H_4-PteGlu$_5$ alone at concentrations greater than 18 μM resulted in marked inhibition of enzyme activity.

TABLE 3.—Effect of pteroylpolyglutamates on tight binding inhibition of thymidylate synthetase by FdUMP

Reactants in preincubation	Enzyme activity units/ml	Percent activity
E	1.46 ± 0.48*	100
E+5-FdUMP	1.41 ± 0.45	97
E+(ℓ)5,10-CH_2H_4PteGlu$_5$ (10 μM)	1.41 ± 0.77	97
E+(ℓ)5,10-CH_2H_4PteGlu$_5$ (5 μM)+FdUMP	0.91 ± 0.23	62
E+(ℓ)5,10-CH_2-H_4PteGlu$_5$ (10 μM)+FdUMP	0.37 ± 0.22	25
E+7,8-H_2PteGlu$_5$ (18 μM)	1.17 ± 0.27	80
E+7,8-H_2PteGlu$_5$ (9 μM)+FdUMP	1.12 ± 0.15	77
E+7,8-H_2PteGlu$_5$ (18 μM)+FdUMP	0.72 ± 0.01	49

*Mean ± SD (N=3).

Thymidylate synthetase at concentration of 18 nM (specific activity 55 units/mg protein) was preincubated for 10 minutes at 37 °C with either 5,10-CH_2-H_4PteGlu$_5$ in buffer C or 7,8-H_2PteGlu$_5$ in buffer D in the presence or absence of 20 nM FdUMP. Five μl aliquots were removed and assayed for 45 minutes at 37 °C in a volume of 105 μl.

Since both 5,10-CH_2-H_4PteGlu$_5$ and H_2PteGlu$_5$ stimulated very tight binding of FdUMP to thymidylate synthetase, it was of interest to determine if such complexes were stable to polyacrylamide gel electrophoresis. Gel electrophoresis under nondenaturing conditions has permitted the separation of multiple ternary complexes of thymidylate synthetase having varying enzyme FdUMP-ratios, and presumably net charges (20). Figure 3A illustrates the pattern obtained after electrophoresis of a mixture containing thymidylate synthetase and a sixfold molar excess of [^{3}H]-FdUMP. FdUMP and thymidylate synthetase did not form a binary complex that was detectable by electrophoresis. The only significant peak of radioactivity recovered from the gel represented unbound FdUMP migrating with an R_f of 1.0 relative to the bromophenol blue marker. The addition of MTX(Glu_1) to the [^{3}H]-FdUMP-thymidylate synthetase reaction mixture did not result in a stable ternary complex being formed (fig 3B). On the other hand, 7,8-H_2PteGlu$_5$ and 5,10-CH_2H_4PteGlu$_5$ formed electrophoretically stable ternary complexes with thymidylate synthetase and FdUMP, as evidenced by a major radioactivity peak (R_f 0.71) of approximately equal area in each gel (figs 3C and 3D).

DISCUSSION

The studies presented here support the proposal that the sequence-dependent effects of MTX and FUra on tumor cell kill may be mediated through an MTX-induced accumulation of dihydrofolate polyglutamates. H_2PteGlu$_5$ induced the formation of very

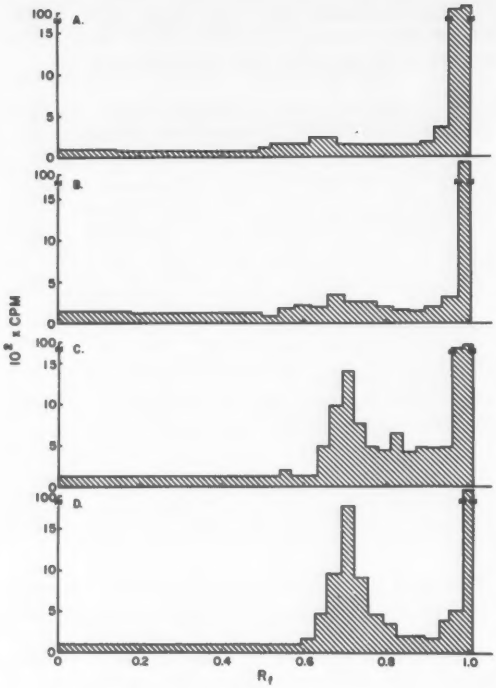

FIGURE 3.—Polyacrylamide native gel electrophoresis of ternary complexes of thymidylate synthetase. Thymidylate synthetase in buffer A at a concentration of 83 nM (specific activity 139 units/mg protein) was incubated for 10 minutes at 37 °C with 500 nM [^{3}H]-FdUMP in either the absence of cofactor (4A), or in the presence of either 10 μM MTX(Glu_1) (4B), 4 μM 7,8-$H_2PteGlu_5$ (4C), or 10 μM 5,10-CH_2-$H_4PteGlu_5$ (4D). Electrophoresis was performed as described in METHODS.

tight complexes with FdUMP and thymidylate synthetase, which could counteract the potentive effect of MTX in inhibiting FdUMP binding by lowering the intracellular levels of tetrahydrofolates.

According to the mathematical model proposed by Jackson and Harrap (19) and the results of Nixon et al (18), the concentration of 7,8-$H_2PteGlu_{(n)}$ could increase by two orders of magnitude as a result of inhibition of dihydrofolate reductase by MTX, becoming the predominant intracellular folate after a 1 μM MTX exposure. At this high intracellular concentration, dihydropteroylpolyglutamates could be important cofactors regulating the binding of FdUMP to thymidylate synthetase. Consistent with this hypothesis were the findings that: (a) 7,8-$H_2PteGlu_5$ acted with FdUMP to produce 51% inhibition of enzyme activity; (b) ternary complexes formed with 7,8-$H_2PteGlu_5$ were stable to the dissociating forces of electrophoresis. However, the partially reduced state of $H_2PteGlu_5$ would be expected to preclude formation of methylene bridge between the folate and the five position of FdUMP, thus preventing a covalent ternary complex from being formed (21).

The less than additive effect on cell viability when cells were exposed to 5 FUra prior to MTX may be related to both the buildup of dUMP (22), and the decreased rate of depletion of the tetrahydrofolate pools resulting from inhibition of thymidylate synthetase by FdUMP. Myers et al (23) have shown that dUMP at concentrations from 10^{-6}M to 10^{-4}M progressively slowed the rate of inactivation of partially purified thymidylate synthetase by FdUMP.

We believe that the results presented here could have direct implications to the mode of action of the antitumor drug, FUra, when it is used either alone or in the clinically important FUra-MTX combination. Based on the data of Moran et al (6) and assuming 0.68 ml of intracellular water per 10^9 cells, the total folate pool in L1210 cells can be calculated as approximately 8 μM, with combined $H_4PteGlu_n$ and 5,10-CH_2-$H_4PteGlu_n$ pools at 2 μM. However, 5 μM 5,10-CH_2-$H_4PteGlu_5$ resulted in only 37% inhibition of thymidylate synthetase by FdUMP (table 3). Thus, it is possible that the intracellular levels of 5,10-CH_2-$H_4PteGlu$ are insufficient for maximal FdUMP binding in some cells. Indeed, Ullman et al have shown that the folate requirement for optimal growth of L1210 cells is less than that required for maximal cytotoxicity of 5-fluoro-2′-deoxyuridine (24).

MTX pretreatment has also been noted to increase the conversion of FUra into FdUMP, as well as to enhance the incorporation of FUra into RNA (2). The relative role of the latter event in relation to the enhanced cell kill observed is not clear, but one or both mechanisms may be acting in various cells to cause enhanced cell kill.

REFERENCES

1. CADMAN E, DAVIS L, and HEIMER R. Sequence, time and dose dependency of methotrexate's enhancement of 5-fluorouracil entry and subsequent cell death of L1210 leukemia. Proc Am Assoc Cancer Res 19:211, 1978.
2. CADMAN E, HEIMER R, and DAVIS L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation of drug synergism. Science 205:1135–1137, 1979.
3. BERTINO JR, SAWICKI WL, LINDQUIST CA, ET AL. Schedule dependent antitumor effects of methotrexate and 5-fluorouracil. Cancer Res 37:327–328, 1977.
4. BROWN I, and WARD HWC. Therapeutic consequences of antitumor drug interactions: methotrexate and 5-fluorouracil in the chemotherapy of C3H mice with transplanted mammary adenocarcinoma. Cancer Lett 5:291–297, 1978.
5. MARTIN DS, HAYWORTH DE, STOLFI R, ET AL. Methotrexate activity against human breast cancer correlates with positive activity against a spontaneous murine ($CD8F_1$) mammary cancer. Proc Am Assoc Cancer Res 17:130, 1976.
6. MORAN RG, WERKHEISER WC, and ZAKRZEWSKI SF. Folate metabolism in mammalian cells in culture. I. Partial characterization of the folate derivatives present in L1210 mouse leukemia cells. J Biol Chem 251:3569–3575, 1976.
7. BAUGH CM, KRUMDIECK CL, and NAIR MG. Polygammaglutamyl metabolites of methotrexate. Biochem Biophys Res Commun 52:27–34, 1973.

8. Coward JK, Parameswaran KN, Cashmore AT, et al. 7,8-dihydropteroyl oligo-γ-L-glutamates: synthesis and kinetic studies with purified dihydrofolate reductase from mammalian sources. Biochemistry 13:3899–3903, 1974.
9. Chu MY, and Fischer GA. The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemic cells (L5178Y). Biochem Pharmacol 17:753–767, 1968.
10. Rode W, Scanlon KJ, Hynes J, et al. Purification of mammalian tumor (L1210) thymidylate synthetase by affinity chromatography on stable biospecific adsorbent. Stabilization of the enzyme with neutral detergents. J Biol Chem 254:11538–11543, 1979.
11. Bradform MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, 1976.
12. Roberts D, and Loehr E. Elevation of thymidylate synthetase activity in CCRF-CEM cells. Cancer Res 31:1181–1187, 1971.
13. Dolnick BJ, and Cheng Y-C. Human thymidylate synthetase II: derivations of pteroylmono and polyglutamates as substrates and inhibitors. J Biol Chem 253:3563–3567, 1978.
14. Kamen BA, Cashmore AR, Dreyer RN, et al. Effect of [^{3}H]methotrexate impurities on apparent transport of methotrexate by a sensitive and resistant L1210 cell line. J Biol Chem 255:3254–3257, 1980.
15. Davis BJ. Disc electrophoresis-II. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427, 1964.
16. Dolnick BJ, and Cheng Y-C. Human thymidylate synthetase derived from blast cells of patients with acute myelocytic leukemia. Purification and characterization. J Biol Chem 252:7697–7703, 1977.
17. Houlinhan CM, and Scott JM. The identification of pteroylpentaglutamate as the major folate derivative in rat liver and the demonstration of its biosynthesis from exogenous [^{3}H]pteroylglutamate. Biochem Biophys Res Comm 48:1675–1681, 1972.
18. Nixon PF, Slutsky G, Nahas A, et al. The turnover of folate coenzymes in murine lymphoma cells. J Biol Chem 248:5932–5936, 1973.
19. Jackson RA, and Harrap KR. Studies with a mathematical model of folate metabolism. Arch Biochem Biophys 158:827–841, 1973.
20. Aull JL, Lyon JA, and Dunlap RB. Gel electrophoresis as a means of detecting ternary complex formation of thymidylate synthetase. Microchem J 19:210–218, 1974.
21. Danenberg PV, Langenbach RJ, and Heidelberger C. Structures of reversible and irreversible complexes of thymidylate synthetase and fluorinated pyrimidine nucleotides. Biochemistry 13:926–933, 1974.
22. Klubes P, Connelly K, Cerna I, et al. Effects of 5-fluorouracil on 5-fluorodeoxyuridine 5′-monophosphate and 2-deoxyuridine-5′monophospate pools, and DNA synthesis in solid mouse L1210 and rat Walker 256 tumors. Cancer Res 38:2325–2331, 1978.
23. Myers CE, Young RC, and Chabner BA. Biochemical determinants of 5-fluorouracil response in vivo. J Clin Invest 56:1231–1238, 1975.
24. Ullman B, Lee M, Martin DW, et al. Cytotoxicity of 5-fluoro-2′-deoxyuridine: requirement for reduced folate cofactors and antagonism by methotrexate. Proc Natl Acad Sci 75:980–983, 1978.

Methotrexate with Thymidine, Inosine, and Allopurinol Rescue: A Phase I Clinical Study

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SUMMARY

A phase I clinical study of a combination of thymidine (TdR), inosine (IR), and allopurinol used as rescue from 24-hour infusions of methotrexate (MTX) was undertaken following animal studies that had shown a better preservation of antitumor activity with this system than with folinic acid. TdR and IR were given in the dose ratio 5:1 by weight throughout. Rescue from MTX, 400 mg/m², could be achieved with a TdR dose in the combination of 1 g/m²/24 hours. This same rescue was also effective when the MTX dose was increased to 800 mg/m², but only partly effective at an MTX dose of 1.5 g/m². It was not necessary to raise the levels of circulating nucleosides significantly above normal to achieve rescue. MTX-related skin lesions occurred more frequently than might be expected with the MTX dose used.

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Methotrexate (MTX) inhibits the enzymic reduction of dihydrofolate to tetrahydrofolate, thereby causing depletion of intracellular pools of the tetrahydrofolate cofactors required for the de novo synthesis of thymidylate and purine ribonucleotides (1,2). Lack of both purine and thymidine (TdR) ribonucleotides contributes to cell kill when L5178Y cells in culture are exposed to MTX (3). Different requirements for TdR and purine rescue have been shown in five cultured cell lines treated with MTX (4). In the mouse, MTX produces a rapid depletion of thymidylate in gut and bone marrow; a purineless state is rapidly established in the gut, but develops more slowly in bone marrow (5). This rapid depletion of purine in the gut is possibly due to the high level of xanthine oxidase activity in this tissue (6).

Potentially lethal doses of MTX with folinic acid (FA) rescue are used in anticancer chemotherapy. FA effectively repletes the intracellular tetrahydrofolate pools, depleted by the action of MTX, but must compete with MTX for a common membrane transport system (7,8) in order to enter the cell.

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Studies have been reported in which it was found to be possible to rescue mice from MTX using TdR in high doses (9). Further, Grindey et al found that the survival of TdR-rescued tumor-bearing animals was better than those rescued with FA, suggesting that TdR may have selectively rescued the normal tissues of the animals (10). In contrast, our own studies of mice bearing the L1210 tumor indicated that the provision of a purine supply was necessary in addition to TdR for total reversal of MTX toxicity. Nevertheless, the antitumor results with such a rescue were better than those found when FA rescue was used (5,6,11).

These observations suggested that a difference may exist between the ability of tumor and normal tissues to utilize salvage metabolites of nucleic acids. Human studies have shown that high doses of TdR can be used as rescue from MTX (12–15).

We have carried out a phase I clinical study of a combination of inosine (IR), TdR, and allopurinol (ITA, in combination) used to rescue normal tissues after an MTX infusion. The study was carried out in three parts: first, any possible toxicity from the ITA given without MTX was investigated; second, the minimum dose of ITA required for rescue after a standard 24-hour infusion of MTX was established; and third, the effectiveness of low-dose ITA rescue with increasing doses of MTX was studied.

It was also possible to measure both the normal and pharmacological levels of various purine and pyrimidine derivatives during this study.

PATIENTS AND METHODS

Patients

Twenty-eight patients at the Royal Marsden Hospital, with histologically proven carcinoma for which no effective conventional treatment was available, were given a total of 32 nucleoside infusions. Ages ranged from 42 to 72 years, mean 60 years; 24 were male and 4 were female. Eighteen had squamous cell carcinoma, five had adenocarcinoma, two had small cell carcinoma of the bronchus, two had carcinoma of the bladder, and one had an adenocarcinoma of the colon. Seventy-three percent had undergone previous chemotherapy. Entry criteria for the study were: a life expectancy greater than 2 months; EDTA clearance > 60 ml/minute; plasma urea $< 7.5 \times 10^{-3}$M; plasma urate $< 0.5 \times 10^{-3}$M; wbc $> 3 \times 10^{9}$/liter; and platelets $> 100 \times 10^{9}$/liter. Patients with markedly abnormal liver function tests were excluded. Informed consent was obtained from all patients.

Nucleoside Infusions

Analytical grade IR and TdR (Cambrian Chemicals Ltd.) were prepared in saline solution, pH 8.5, adjusted to be isotonic with human plasma. Solutions were sterilized by autoclave, after which process high performance liquid chromatograms (HPLC) showed 2% conversion of TdR to thymine, but no other effect on the nucleosides. Nucleoside infusions were prepared in the pharmacy of the Royal Marsden Hospital.

Study Design and Treatment Plan

Each group of three patients received the same treatment (table 1). MTX was given in 1 liter of physiological saline over 24 hours, followed immediately by the rescue infusion, which was given in 2 liters of saline over 48 hours, unless otherwise stated. Allopurinol, 200 mg every 8 hours, was given by mouth throughout the rescue period. It has been established that TdR 8 g/m²/day is well tolerated and is an effective rescue from high MTX doses (13). Our animal studies indicated that the optimum weight ratio of TdR to IR for rescue was 5:1 (16); thus TdR 8 g/m²/day and IR 1.6 g/m²/day was designated the maximum rescue. In the past, hypoxanthine has been given to human volunteers (17), but at a much lower dose than that required for maximum rescue; therefore, in part 1 of this study IR was increased, in stages, to 1.6 g/m²/day with the fixed TdR dose of 8 g/m²/day. In part 2, the rescue, given after 400 mg/m²/24 hours MTX, was decreased, with the TdR:IR ratio maintained at 5:1, until MTX toxicity was observed. A minimum safe rescue was then selected; this was the rescue dose that was twice that at which MTX toxicity was first seen. In part 3, the dose of MTX in the 24-hour infusion was increased first to 800 mg/m², and then to 1,500 mg/m², with the same minimum safe rescue, which was infused until the circulating MTX level had fallen below 6×10^{-8}M.

TABLE 1.—Treatment schedules and toxicity

Part	Number of patients	MTX mg/m²/24 hours	IR g/m²/24 hours	TdR g/m²/24 hours	Toxicity	
					Number of toxic courses	Patient* initials
1	3	-	0.4	8.0	0	
	3	-	0.8	8.0	0	
	3	-	1.6	8.0	0	
2	3	400	1.6	8.0	0	
	3	400	0.8	4.0	0	
	3	400	0.4	2.0	0	
	2	400	0.2	1.0	0	
	3	400	0.1	0.5	1	W.M.
	3	400	0.05	0.25	1	Z. B.
3	3	800	0.2	1.0	0	A. T.
	3	1,500	0.2	1.0	2	W. B.

Allopurinol, 200 mg every 8 hours was given orally during all infusions.
*Details of toxicity are under these initials in the RESULTS section.

Plasma MTX levels were measured by the method of Bertino and Fischer (18), and circulating nucleosides and bases were measured by an HPLC method developed in our laboratory by G. A. Taylor (19).

RESULTS

Circulating Nucleosides and Bases

Purines.—Plasma hypoxanthine levels were measured in 13 healthy volunteers: these ranged from 0.5 μM to 2.8 μM (mean 1.62 μM, SEM ± 0.20 μM). Twenty-five cancer patients in whom circulating hypoxanthine levels were measured before treatment fell into two distinct groups, a low group of 20 patients in whom the values fell between 0.85 μM and 6.5 μM (mean 2.62 μM ± 0.32 μM) and a high group of 5 patients, range 49.8 μM to 64.2 μM (mean 52.4 μM ± 2.3 μM). Comparison of mean circulating hypoxanthine levels in healthy volunteers and the low group of patients shows a significant difference ($P < 0.01$). ITA infusions produced changes in circulating hypoxanthine concentration (fig 1); if the initial plasma level was in the low range the increase was related to the amount of IR infused, whereas in the high group ITA infusion gave no increase in circulating hypoxanthine.

In 14 normal subjects, IR levels were found to lie between 0.26 μM to 1.56 μM (means 0.68 μM ± 0.09 μM). Accurate measurement of plasma IR during rescue infusion was not possible, due to interference by a compound that was probably allopurinol riboside (allopurinol was given throughout the rescue infu-

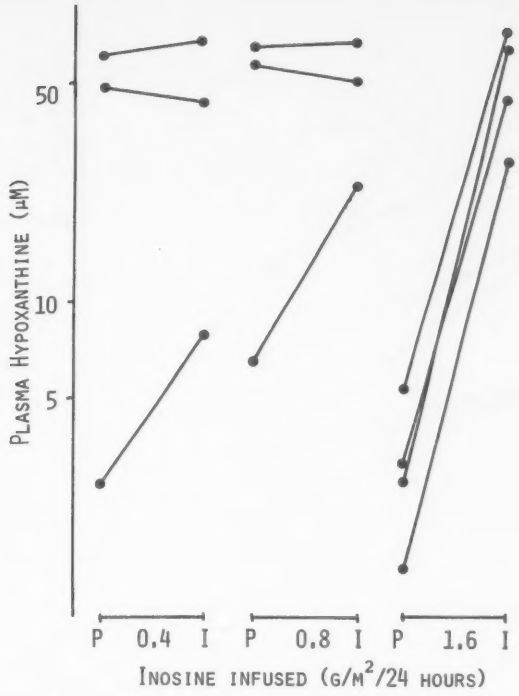

FIGURE 1.—Changes in plasma hypoxanthine during rescue measured by HPLC before any treatment (P) and during the plateau of the rescue infusions (I). The IR dose was escalated in three stages (0.4, 0.8, and 1.6 g/m²/24 hours) with a constant dose of TdR (8 g/m²/24 hours), and allopurinol 200 mg every 8 hours. Each line shows the change in an individual patient. The hypoxanthine levels are plotted on a logarithmic scale.

sion). Plasma concentrations of allopurinol and oxypurinol, respectively > 1 μM and > 50 μM, were maintained during hypoxanthine half-life measurements (table 2). Doses of 1.6 g of allopurinol given by mouth over 48 hours gave a range of plasma allopurinol levels from 1.6 μM to 45.4 μM (mean 16.9 μM) and oxypurinol from 43 μM to 199 μM (mean 106 μM). Table 2 shows the plasma half-lives. Plasma urate fell during ITA administration (fig 2).

Pyrimidines.—In plasma samples from 12 healthy volunteers, a range of TdR concentration from 0.10 μM to 0.9 μM (mean 0.43 μM ± 0.063 μM) was observed. Twelve pretreatment samples from patients were chromatographed: in five of these the TdR level was below that which was accurately measurable by our method (0.1 μM); the median value was 0.17 μM, and the highest was 2.0 μM. The plasma TdR concentrations achieved during rescue infusions are shown in figure 3, and plasma half-life in table 2. Variable thymine concentrations were found in plasma during

TABLE 2.—Plasma half-lives of some nucleosides and bases

	Half-life (minutes)
Hypoxanthine	75 to 100
Allopurinol	75
Oxypurinol	>120
TdR	15

Half-lives were measured by HPLC following rescue infusions. Allopurinol and oxypurinol were circulating at high levels during the determination of hypoxanthine half-life.

infusions. Thymine levels were measured after the infusions were discontinued, and remained constant until the TdR level had fallen below 10 μM.

Clinical Toxicity of ITA Rescue Alone (Part 1)

In three cases the maximum rescue infusion caused phlebitis in the peripheral vein in which the infusion was sited. One patient complained of dizziness during a rescue infusion, but there were no neurological signs and the episode passed quickly.

MTX Toxicity with ITA Rescue (Parts 2 and 3)

The results of the MTX toxicity with ITA rescue (parts 2 and 3) are summarized in table 1. With the MTX dose of 400 mg/m², toxicity was not seen until the dose of TdR in the rescue infusion fell below 1 g/m²/day. The minimum safe rescue was thus defined as TdR 1 g/m²/day, with IR 0.2 g/m²/day and allopurinol. Rescue infusions at this level produced no measurable change in the circulating levels of TdR or hypoxanthine. Escalation of MTX dosage to 800 mg/m² with this rescue did not result in toxicity. However, toxicity was seen when the MTX dose was further increased to 1.5 g/m².

MTX infusions of 400 mg/m²/day gave plasma concentrations of between 0.2 μM and 2 μM. Figure 4 shows the levels achieved with higher MTX doses and the plasma decay curves after infusion.

Toxicity: Case Reports

Four patients showed definite evidence of MTX toxicity:

W.M.—A man of 68 who, 4 years before treatment, had developed peripheral neuropathy and was found to have a 1 gM monoclonal band on plasma electrophoresis. Waldenström's macroglobulinemia was diagnosed following a bone marrow aspiration. Two years later an inoperable squamous cell carcinoma of the bronchus was found, and subsequently six courses of ifosfamide were given. At the time of MTX/ITA treatment, intrapulmonary disease was extending. EDTA clearance was 63 ml/minute (normal range

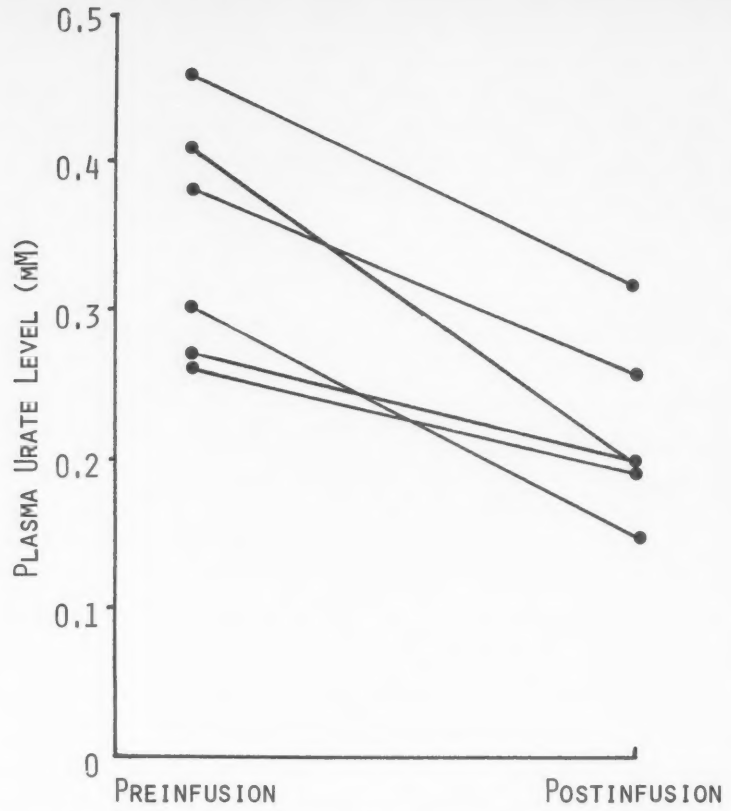

FIGURE 2.—Plasma urate levels taken before ITA rescue infusion and immediately after rescue had been discontinued in six patients.

110 to 125 ml/minute), just adequate for entry into the study. MTX, 400 mg/m² was given, followed by IR 0.1 g/m²/day and TdR 0.5 g/m²/day. During the rescue the patient developed glucosuria with normal blood sugar, but did not become uremic. Eight days from the start of the infusion a transitory maculopapular rash developed. By 13 days a marked neutropenia had developed (0.2×10^9 neutrophils/liter) and the patient died of bronchopneumonia.

Z.B.—A 66-year-old man who had also been found to have a 1 gM monoclonal band in the plasma, but the EDTA clearance was within the normal range. Shortly before treatment, carcinoma of the colon had been diagnosed, with metastases in bone and liver. MTX 400 mg/m² was followed by IR 0.05 g/m²/day and TdR 0.25 g/m²/day for 48 hours. During the rescue acute urinary retention developed, then intractable hiccoughs. The patient became drowsy and disoriented. Urea remained normal. Five days after MTX a rash developed, and at 6 days the patient became neutropenic and pyrexial. FA 60 mg was given iv (19), and the patient's condition improved. The blood count returned to normal by 19 days from MTX administration.

A.T.—This 43-year-old man with adenocarcinoma of the bronchus was previously treated by pneumonectomy, then with radiotherapy and combination chemotherapy. MTX 1.5 g/m² was given, and a total dose of IR 0.5 g and TdR 2.5 g was given over 60 hours. The decay of plasma MTX is shown in figure 4. The plasma level fell comparatively slowly; 48 hours after MTX infusion was finished the plasma level was 1×10^{-7} M, and at 52 hours was 6×10^{-8} M. Seven days after MTX infusion an extensive rash developed; at 10 days, severe oral mucositis; at 14 days, neutropenia (1.5×10^9 neutrophils/liter) and thrombocytopenia (22×10^9 platelets/liter) developed. Thereafter, the patient made a slow recovery, and 21 days after MTX the blood count had returned to normal.

W.B.—A 63-year-old man who had squamous cell carcinoma of the bronchus, with bilateral involvement of supraclavicular and cervical nodes, compli-

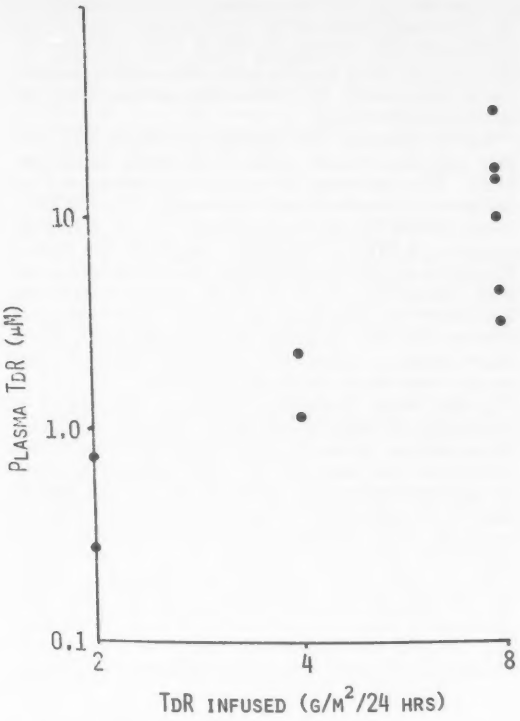

FIGURE 3.—Plasma TdR levels, measured by HPLC, achieved with rescue infusions of 2, 4, and 8 g/m²/24 hours TdR, with purine. The scale is logarithmic. Each point represents a value from one patient.

cated by longstanding mitral incompetence. EDTA clearance was 96 ml/minute and the other criteria for entry to the study were met. MTX 1.5 g was given over 24 hours, followed by a total dose of IR 0.6 g and TdR 3.0 g given over 72 hours until the plasma level of MTX was $< 1\times10^{-8}$ M. Twenty-four hours after the rescue was terminated, a severe rash developed. The patient became thrombocytopenic and neutropenic 7 days after MTX and was then given 160 mg FA. Fifteen days after MTX, the blood count began a rapid recovery, and returned to normal 18 days after MTX.

DISCUSSION

Circulating Nucleosides and Bases

Leyva and coworkers (15) measured circulating hypoxanthine, IR and xanthine in normal subjects and pretreatment patients. They found hypoxanthine levels ranging from 1 μM to 4 μM, similar to our results in normal subjects and patients in the low hypoxanthine range. We found a significant difference in the mean levels of hypoxanthine in patients and healthy volunteers; possibly this is caused by increased cell breakdown in patients with advancing cancer. Leyva also reported that there was a group of patients with circulating purine levels up to 100 μM, which is in agreement with our findings, suggesting either an exceptionally rapid breakdown of tissue in these patients or an inability to salvage, catabolize, or excrete purine. Clinically these patients did not as a group differ from other patients, but their numbers were small. During rescue infusions the plasma level of hypoxanthine rose rapidly except in the high pretreatment hypoxanthine group where the infused purine was probably insignificant in comparison with the endogenous hypoxanthine. We conclude that, in the human, IR is rapidly converted to hypoxanthine, which is not further metabolized to urate in the presence of allopurinol and oxypurinol.

Normal human serum TdR levels, measured by microbioassay and radioimmunoassay, have been report-

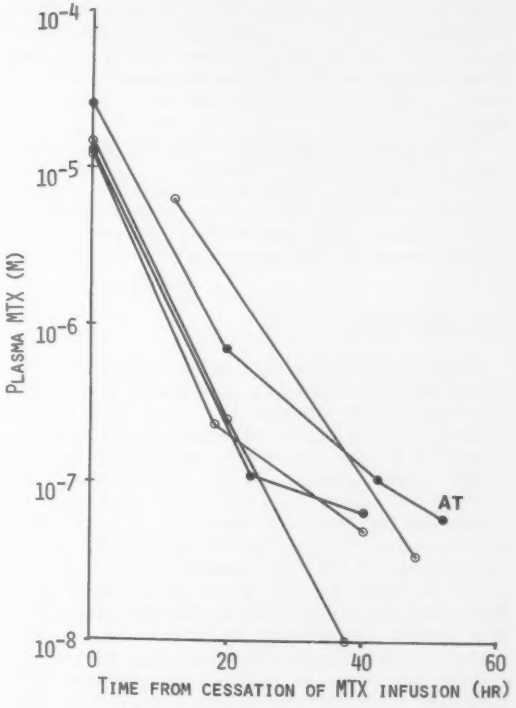

FIGURE 4.—MTX plasma decay. The circulating levels of MTX were measured after 24-hour infusions of 800 mg/m² (○) or 1,500 mg/m² (●) of MTX, and are plotted on a logarithmic scale. A. T. was a patient who developed evidence of MTX toxicity.

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ed as 0.041 μM to 0.087 μM and 0.1 μM to 0.76 μM respectively (21,22). With an HPLC system (15), a mean TdR plasma level of 0.35 μM ± 0.17 μM has been reported. Our results were of a similar order, but indicated considerable individual variation. Patients vary widely in their tolerance of unrescued MTX (22), and this tolerance may be at least partially influenced by the availability of salvageable nucleic acid metabolites.

Toxicity: Part 2

Toxicity of MTX is related to both the dose and the time over which the dose is given. Patients given MTX 400 mg/m² over 24 hours were all rescued by TdR > 1 g/m²/day concomitant with IR and allopurinol. Half this dose produced symptom-free rescue in two out of three patients, although one patient with preexisting renal insufficiency died of the effects of MTX toxicity. When TdR was reduced to 0.25 g/m²/day and IR to 0.05 g/m²/day, all MTX toxicity was still averted in one of three patients. Howell et al (13) found that TdR alone, 1 g/m²/day, given 24 hours after a bolus injection of MTX 3 g/m², was not sufficient to prevent mucositis in 34% of patients. Direct comparison of these findings with our results is difficult because of the differing dose of MTX and mode of administration, but this does suggest that the addition of purine to TdR adds to the effectiveness of the rescue.

At the minimum safe rescue (TdR 1.0, IR 0.2 g/m²/day) no change could be detected in the circulating levels of TdR, thymine, or hypoxanthine. It has been observed (23) that even in the presence of 10 μM MTX, human bone marrow cells are protected by small increases in TdR concentration over the 0.1 μM level.

Toxicity: Part 3

When the MTX infusion was increased to 800 mg/m²/24 hours the minimum safe rescue dose of ITA was found to be effective, but when MTX was increased to 1,500 mg/m²/24 hours, toxicity was encountered. In this part of the study rescue was continued until plasma MTX concentration was $<6\times10^{-8}$ M, considered a safe MTX level when FA rescue has been used (24). Since FA and MTX compete for cellular uptake it may be expected that in the presence of FA the intracellular level of MTX would be lower for any given circulating level of MTX than if nucleoside rescue had been given. Thus it is possible that although nucleoside rescue rapidly restarts DNA synthesis (25), after it has been discontinued there could be sufficient intracellular MTX persisting to inhibit DNA synthesis again. This could give rise to a further, delayed episode of MTX toxicity even though the rescue was discontinued at an MTX level considered safe when FA rescue is employed. In two patients (A. T. and W. M.) peripheral evidence of bone marrow suppression was not seen for 2 weeks after MTX was given. In the final patient (W. B.) treated with MTX 1,500 mg/m², rescue was continued until circulating MTX was $<1\times10^{-8}$ M, but even so, MTX toxicity occurred, possibly indicating a requirement for nucleosides greater than the minimum safe rescue.

All four patients who showed evidence of MTX toxicity had skin rashes, which were severe in two patients. The rationale for nucleoside rescue is that different tissues (both host and tumor) differ in their requirements for nucleic acid precursors, following exposure to MTX. It has been suggested (24) that if FA rescue is given, skin lesions do not usually occur until circulating levels of MTX exceed 1×10^{-6} M. This level was not reached in any of our patients. Possibly the skin is relatively less able than many other tissues to utilize nucleosides, and is particularly at risk when this form of rescue is used.

In this study allopurinol was given as a means of increasing salvageable purine by inhibition of xanthine oxidase. Metabolites of allopurinol also inhibit orotidylate decarboxylase (26), and thus may decrease de novo synthesis of all pyrimidines. At the dose of allopurinol used there was no evidence of toxicity from this cause. Recently there has been interest in TdR as a potentially cytotoxic drug (27–30). It has been suggested that this action is mediated by distortion of intracellular deoxyribonucleotide pools (29), although in order for cytotoxicity to occur it is necessary for TdR concentration to exceed 10^{-3} M, a level considerably higher than those reached in this study.

CONCLUSIONS

We conclude that MTX toxicity may be ameliorated by ITA rescue. It is possible that the addition of the purine derivatives may reduce the requirement for TdR in the rescue. This might increase the likelihood of achieving a selective rescue directed towards normal tissues. However, more hospitalization and supervision of patients is needed than when FA rescue is used, which adds to the cost and detracts from the acceptability of the procedure.

We suggest that future studies might be profitably conducted in selected patients with tumors known to have activities of those enzymes that degrade nucleosides or low activities of the appropriate activating enzymes. One example of such an approach would be well-differentiated hepatoma in which the enzyme dihydrothymine dehydrogenase is preserved (31).

REFERENCES

1. Mead JAR, Venditti JM, Schrecker AW, et al. The effect of reduced derivatives of folic acid on toxicity and antileukaemic effect of methotrexate in mice. Biochem Pharmacol 12:371, 1963.
2. Werkheiser WC. The biochemical, cellular and pharmacological activation, and effects of the folic acid antagonists. Cancer Res 23:1277, 1963.

3. Hryniuk WM. The mechanism of action of methotrexate in cultured L5178Y leukaemia cells. Cancer Res 35:1085, 1975.
4. Tattersall MHN, Jackson RC, Jackson STM, et al. Factors determining cell sensitivity to methotrexate: studies of folate and deoxyribonucleoside triphosphate pools in five mammalian cell lines. Eur J Cancer 10:819, 1974.
5. Straw JA, Talbot DC, Taylor GA, et al. Some observations on the reversibility of methotrexate toxicity in normal proliferating tissues. J Natl Cancer Inst 58:91, 1977.
6. Dady PJ. Enhancement of antifolate cancer chemotherapy. M. D. Thesis, University of London, 1979, p 31.
7. Goldman ID, Lichtenstein NS, and Oliverio VT. Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukaemia cell. J Biol Chem 19:5007, 1968.
8. Nahas A, Nixon PF, and Bertino JR. Uptake and metabolism of N^5-formyltetrahydrofolate by L1210 leukaemia cells. Cancer Res 32:1416, 1972.
9. Tattersall MHN, Brown BL, and Frei E (iii). The reversal of methotrexate toxicity by thymidine, with maintenance of antitumour effects. Nature 253:198–200, 1975.
10. Grindey GB, Semon JH, and Pavelic ZP. Modulation versus rescue of antimetabolite toxicity by salvage metabolites administered by continuous infusion. Antibiot Chemother 23:295, 1978.
11. Harrap KR, Taylor GA, and Browman GP. Enhancement of the therapeutic effectiveness of methotrexate and protection of normal proliferating tissues with purines and pyrimidines. Chem Biol Interact 18:119, 1977.
12. Howell SB, Krishan A, Frei E. Cytokinetic comparison of thymidine and leucovorin rescue of marrow in humans after exposure to high dose methotrexate. Cancer Res 39:1315, 1979.
13. Howell SB, Ensminger WD, Krishan A, et al. Thymidine rescue of high-dose methotrexate in humans. Cancer Res 38:325, 1978.
14. Howell SB, Herbst K, Boss G, et al. Thymidine (TdR) rescue of methotrexate (MTX) in man. Proc Am Soc Clin Oncol 20:399, 1979.
15. Leyva A, Weiss R, Schornagel JH, et al. Plasma levels of pyrimidines and purines during methotrexate (MTX)-thymidine (TdR) treatment. Proc Am Assoc Cancer Res 20:250, 1979.
16. Dady PJ. Enhancement of antifolate cancer chemotherapy. M. D. Thesis, University of London, 1979, p. 41.
17. Goldfinger S, Klinenberg JR, and Seegmiller JE. The renal excretion of oxypurines. J Clin Invest 44:623, 1965.
18. Bertino JR, and Fischer GA. Techniques for the study of resistance to folic acid antagonists. Methods Med Res 10:297, 1966.
19. Taylor GA, Dady PJ, and Harrap KR. Quantitative high performance liquid chromatography of selected nucleosides and bases in human plasma. J Chromatogr, 1979, submitted.
20. Nottebrock H, and Then R. Thymidine concentrations in serum and urine of different animal species and man. Biochem Pharmacol 26:2175, 1977.
21. Ensminger WD, and Frei E. The prevention of methotrexate toxicity by thymidine infusions in humans. Cancer Res 27:1857, 1977.
22. Hansen HH, Selawry OS, Holland JF, et al. The variability of individual tolerance to methotrexate in cancer patients. Br J Cancer 25:298, 1971.
23. Taetle R, Mendelsohn J, and Howell SB. Nucleoside requirements for the protection of human marrow from methotrexate (MTX). Proc Am Soc Clin Oncol 20:3–9, 1979.
24. Bleyer WA. Methotrexate: clinical pharmacology, current status and therapeutic guidelines. Cancer Treat Rev 4:87, 1977.
25. Pinedo HM, Zaharko DS, Bull JM, et al. The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. Cancer Res 36:4418, 1976.
26. Fox RM, Royse-Smith D, and O'Sullivan WJ. Orotidinuria induced by allopurinol. Science 168:861, 1970.
27. Howell SB, Jenkins R. Evaluation of thymidine (TdR) as a chemotherapeutic agent against human tumour xenografts in nude mice. Proc Am Assoc Cancer Res 20:259, 1979.
28. Lee SS, Giovanella BC, Stehlin JS, et al. Further studies on the long term effects of high dose thymidine infusion (TdR) on human tumours heterotransplanted in nude mice. Proc Am Assoc Cancer Res 20:234, 1979.
29. Woodcock T, Danin L, O'Hehir M, et al. Early clinical and pharmacokinetic evaluation of thymidine therapy in patients with advanced cancer. Proc Am Assoc Cancer Res 20:114, 1979.
30. Chuiten DF, Wiernik PH, Zaharko DS, et al. Phase I-II trial and clinical pharmacology of continuously infused high dose thymidine (HD-TdR). Proc Am Assoc Cancer Res 20:76, 1979.
31. Jackson RC, and Weber G. Enzyme pattern directed chemotherapy. The effects of combinations of methotrexate, 5-fluorodeoxyuridine and thymidine on rat hepatoma cells in vitro. Biochem Pharmacol 25:2613, 1976.

Methotrexate Cytotoxicity: Studies on Its Reversal by Folates and Nucleosides

Albert Leyva,[1,2]* Hubertus Nederbragt,[1] Jan Lankelma,[1] and Herbert M. Pinedo[1,2,3]

SUMMARY

The methotrexate (MTX) metabolite, 7-hydroxymethotrexate, which is potentially capable of influencing MTX rescue by affecting both MTX and reduced folate transport at the cell membrane, has been estimated in the plasma of patients receiving moderate doses (240 mg/m²) of MTX. Forty-eight hours after MTX dosage the level of 7-hydroxymethotrexate exceeded that of MTX by ten to one.

Studies of the utilization of thymidine and inosine by MTX-treated L1210 cells in culture suggest that these cells may be able to utilize these substrates when their extracellular concentration is < 1 μM.

The incorporation of ^{14}C hypoxanthine into the nucleotide pools and the nucleic acids of L1210 cells increases following exposure to MTX. This observation is compatible with the known increased availability of phosphoribosyl pyrophosphate in these cells following MTX treatment.

These observations show ways in which naturally occurring nucleosides as well as metabolites of MTX itself may modulate the toxicity of the drug. Such observations may be relevant to the design of treatment and rescue protocols.

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The study of the reversal of methotrexate (MTX) cytotoxicity has been a very active field of research whose aim has been to develop ways of enhancing the therapeutic effectiveness of MTX. Attempts have been made to modulate the activity of MTX at the metabolic level through the use of reversal agents that may be termed either protective or rescue agents depending on the time of administration. The particular aims of these studies have been primarily to control toxicity to normal host tissues and secondarily to impart selective toxicity to tumor tissues (1). The first aim has been quite successful both experimentally and clinically with the use of Ca leucovorin (dl N^5-formyl tetrahydrofolate). Thymidine (TdR) has recently been demonstrated to also be effective in preventing MTX toxicity in patients (2–4).

Leucovorin (LV) acts as a source of reduced folates to replenish cellular pools depleted by the inhibition of dihydrofolate reductase by MTX. The antagonistic effect of LV on MTX cytotoxicity has been compared in normal mouse bone marrow cells and L1210 leukemia cells in vitro (5,6). LV acts in both cell types in a similar manner and is more effective at low MTX concentrations (less than 10^{-6} M). The competitive interaction observed between MTX and LV (probably through competition for a common membrane transport system [7] and for common enzyme binding sites) would indicate limitations for LV reversal of MTX toxicity, since high concentrations of MTX could be effectively reversed only by high concentrations of LV. There is no clear evidence to suggest selective properties of LV. However, supplying reduced folates during or after MTX does result in differential protection or rescue of normal cells. For example, LV rescue has been shown to enhance the therapeutic index of MTX (8,9).

The effect of MTX on folate metabolism results in an inhibitory effect on the de novo synthesis of thymine and purine nucleotides. The availability of TdR and purines can provide for salvage pathway synthesis of these nucleotides. Our studies on mouse bone marrow cells and L1210 cells in vitro suggest that the reversal of MTX toxicity with TdR and a purine has potential advantages over reversal by LV. Reversal was found to be independent of MTX concentration. This independence would prove useful in the manage-

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ment of toxicity at high doses of MTX. Although the normal and leukemic cells both appeared to have the same TdR concentration requirement (10^{-5} M) for reversal of MTX toxicity, the purine concentration required was higher (10^{-4} M) for L1210 compared to normal cells (10^{-5} M). These latter findings and those reported by others (5,6,10–13) describing variability in TdR and purine requirements for reversal of MTX toxicity in various cell lines suggest that differences in nucleotide synthesis via salvage pathways exist from one cell type to another and that these differences may be exploited for selective control of toxicity. In clinical studies, TdR alone as a protective or rescue agent has been shown to be effective in averting MTX toxicity without significant improvement in antitumor effect (2–4). The administration of purines has not been used because of their apparently sufficient availability in plasma.[4]

We report in this paper recent findings relevant to the reversal of MTX cytotoxicity concerning (a) the potential influence of 7-hydroxymethotrexate (7-OHMTX); (b) the determination of minimal requirements of TdR and purines to provide sufficient nucleotide synthesis; and (c) the role of phosphoribosylpyrophosphate (PRPP) in purine reversal.

Potential Influence of 7-OHMTX on MTX Cytotoxicity and Its Reversal

7-OHMTX has been reported to be formed in man during high-dose MTX treatment (>50 mg/kg) (14). We have examined 7-OHMTX plasma levels after infusion of similar doses of MTX in patients and have found an increasing proportion of 7-OHMTX to MTX with time, which prompted us to study the effect of 7-OHMTX on MTX entry into the cell.[5] ^{3}H-MTX uptake was examined using human KB cells in vitro. 7-OHMTX at 2×10^{-5} M was tested with 10^{-6} M ^{3}H-MTX (such concentrations are representative of those observed in plasma of treated patients). The intracellular level of ^{3}H-MTX increased with time and was maximal after 150 minutes' incubation. The presence of 7-OHMTX reduced the maximal uptake of ^{3}H-MTX by 50%, indicating an inhibitory effect of 7-OHMTX on MTX transport. Using a sensitive high pressure liquid chromatography method for the measurement of MTX and 7-OHMTX in plasma (15), we examined plasma samples of 13 head and neck cancer patients who received moderate doses of MTX (240 mg/m^2 in a 24-hour infusion). Figure 1 shows the 48-hour plasma concentrations of MTX and 7-OHMTX that were of

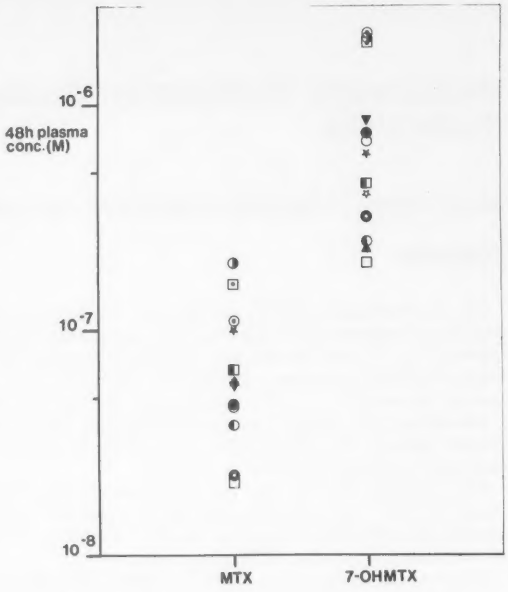

FIGURE 1.—Plasma concentrations of MTX and 7-OHMTX 48 hours after MTX infusion. Plasma samples were obtained from 13 patients with head and neck cancer 48 hours after a 24-hour infusion of MTX at 240 mg/m^2. Measurements were made using high pressure liquid chromatography. A different symbol is used to represent each patient.

the order of 10^{-7} and 10^{-6} M respectively. The mean ratio of 7-OHMTX to MTX was 10.5 ± 4.5 (SD).

The metabolism of MTX to 7-OHMTX has been generally associated with high-dose MTX treatment. However, it appears that the production of 7-OHMTX occurs also at lower, more commonly used doses. The pharmacokinetics of 7-OHMTX itself have just begun to be examined and studies indicate that the 7-OHMTX concentration can exceed that of MTX in plasma, particularly after the end of MTX infusion. This delay in the appearance of elevated plasma levels of 7-OHMTX may be shortened in sequential administration of MTX. So far, there is evidence that 7-OHMTX interferes with the cellular uptake of MTX. It remains to be seen how 7-OHMTX affects the transport of LV or natural reduced folates. The late appearance of significant levels of 7-OHMTX in plasma may have a greater influence on the cellular uptake of reduced folates during the rescue period. The importance of extracellular MTX in inhibiting the uptake of extracellular reduced folates has been indicated (16). If 7-OHMTX has a similar effect on reduced folates it will be necessary to be concerned with both MTX and 7-OHMTX levels in plasma in assessing toxicity of MTX and its reversal by LV.

[4] See Dady et al. Cancer Treat Rep 65 (Suppl 1):37–43, 1981.

[5] Lankelma J, van der Kleijn E, and Jansen MJT, submitted for publication.

TdR and Purine Requirements for Reversal of MTX Cytotoxicity

The results of previous in vitro studies of colony formation by mouse bone marrow cells (5,6) and L1210 mouse leukemia cells (6) in semisolid medium indicated that the normal cells were more efficient than the leukemic cells in overcoming the growth inhibitory effects of MTX via the salvage pathway synthesis of purine nucleotides. The myeloid precursor cells were found to be completely spared from MTX toxicity in the presence of equimolar concentrations of TdR and a purine at 10^{-5} M. We reexamined the question concerning the concentration requirements of TdR and purines for the reversal of MTX toxicity in L1210 cells in liquid culture and found that these cells are capable of substantial utilization of TdR and purines at concentrations below 10^{-5} M.

L1210 cells were cultured and maintained in logarithmic growth in RPMI 1640 medium containing 15% dialyzed fetal calf serum as previously described (6). Cells were subcultured at an initial cell concentration of 5 or 10×10^4 cells/ml and allowed to proliferate for 24 hours before the addition of MTX, TdR, or inosine. Cell counts were performed with a hemacytometer before and for up to 48 hours after these additions. During this 48-hour incubation period cell concentration increased logarithmically in untreated control cultures. MTX alone at 10^{-6} M decreased the cell concentration by more than 90%. Figure 2 illustrates the effect of TdR plus inosine at various concentrations on the MTX-mediated inhibition of cell proliferation when examined at 48 hours after MTX treatment. Maximal reversal of MTX toxicity to L1210 cells was achieved at 10^{-5} M TdR combined with inosine at 10^{-4} M or higher concentrations. Under the latter conditions the resulting cell count was approximately 80% of control, indicating that complete reversal was not achieved. These results were consistent with those of L1210 cells in semisolid medium culture (6).

In order to determine the cause of incomplete prevention of MTX-mediated inhibition of L1210 cell growth by TdR and purines, a more detailed study was performed of the growth characteristics of L1210 cells in the presence of MTX, TdR, and inosine (fig 3). Cell concentration increased to 10 times the initial value in control cultures. In the presence of 10^{-6} M MTX, cell concentration remained stationary for 12 hours but decreased drastically after 24 hours. MTX-treated cultures supplemented with 10^{-4} M inosine plus TdR at 10^{-6}, 3×10^{-6}, and 10^{-5} M continued to grow at control rates for 12, 24, and 36 hours, respectively, before evidence of inhibitory effects. The effectiveness of 10^{-6} M TdR could be prolonged for an additional 12 hours if an extra amount of TdR equivalent of 10^{-6} M was provided at 10 hours after the start of the experiment (fig 4). These results indicate that complete reversal of the growth inhibitory effects of MTX on L1210 cell growth in vitro is possible with TdR at a concentration as low as 10^{-6} M when inosine is present in a high concentration. We are

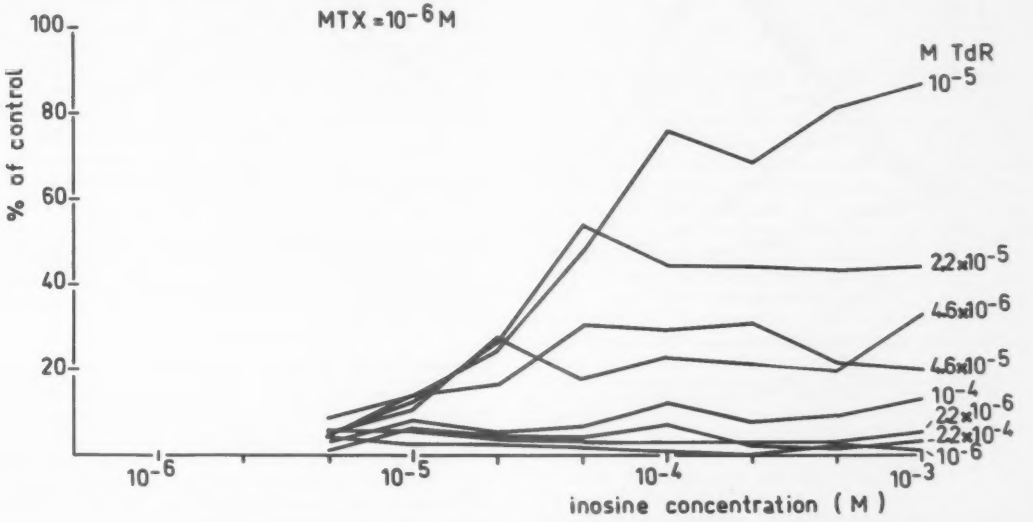

FIGURE 2.—Reversal of MTX toxicity to L1210 cells in vitro by TdR and inosine. Cell concentration was determined 48 hours after the addition of MTX, TdR, and inosine and was expressed as percent of values obtained for 48-hour untreated control cultures.

currently investigating the requirements for purines in the above manner to determine if the limited reversal of MTX cytotoxicity by purines in vitro is due to exhaustion of the purines in the medium.

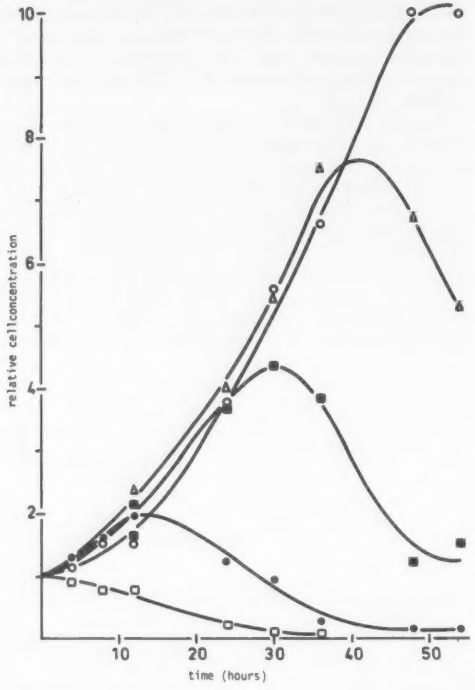

FIGURE 3.—Effect of different concentrations of TdR on MTX toxicity to L1210 cells in the presence of 10^{-4} M inosine. Additions were made when cells were in logarithmic growth and the initial cell concentration was 1.5 to 2.0 $\times$ 10^5 cells/ml. Cell concentration is expressed as times increase over initial values. ○, no additions; □, 10^{-6} M MTX; ●, 10^{-6} M MTX, 10^{-4} M inosine, 10^{-6} M TdR; ■, 10^{-6} M MTX, 10^{-4}M inosine, 3 $\times$ 10^{-6} M TdR; △, 10^{-6}M MTX, 10^{-4} M inosine, 10^{-5} M TdR.

The extent of depletion of nucleosides in the culture medium and the extent of incorporation of nucleosides into cells was studied in L1210 cells over a 24-hour period using ^{14}C-labeled TdR and inosine (tables 1 and 2). Table 1 gives the data for control conditions (in the absence of MTX). At initial concentrations of TdR and inosine in the medium less than or equal to 10^{-6} and 10^{-5} M, respectively, nucleoside concentrations were markedly reduced to 8% to 12%. Only with 5 $\times$ 10^{-6} M TdR and 5 $\times$ 10^{-5} M inosine was the concentration in the medium reduced by only half. For a 24-hour period of logarithmic growth incorporation of TdR and inosine into nucleic acids appeared maximal at 5 $\times$ 10^{-6} and 5 $\times$ 10^{-5} M, respectively. Table 2 shows the results for similar experiments described in table 1 except carried out in the

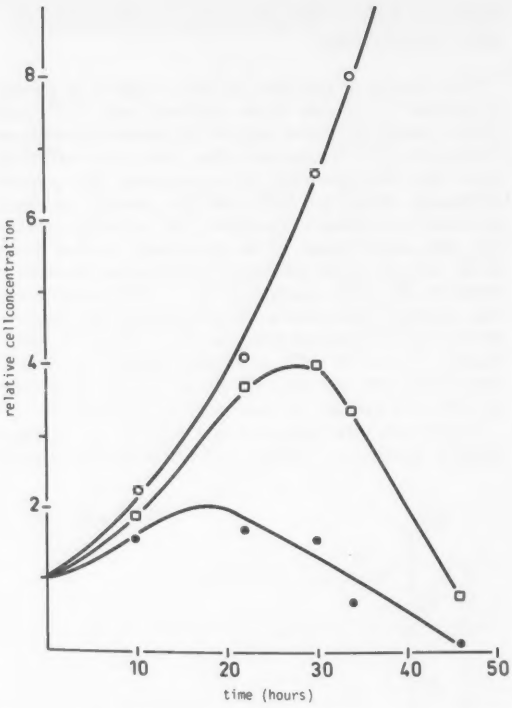

FIGURE 4.—Extended reversal of MTX toxicity at 10^{-4} M inosine and 10^{-6} M TdR. Conditions as in figure 3. ○, no additions; ●, 10^{-6} M MTX, 10^{-4} inosine, 10^{-6} M TdR; □, 10^{-6} M MTX, 10^{-4} M inosine, 10^{-6} M TdR with additional TdR (equivalent to the initial amount of TdR) after 10 hours.

presence of 10^{-7} M MTX. The results are very similar, especially at the higher concentrations of TdR and inosine. At the lower concentrations of inosine (10^{-6} to 10^{-5} M) there appeared to be twice as much inosine remaining in the medium. Tables 1 and 2 also show that in both untreated and MTX-treated cultures with initial cell concentrations of 2 $\times$ 10^{-5} cells/ml the maximal amounts of TdR and inosine incorporated into nucleic acids in 24 hours was 2 nmol and 9 to 10 nmol, respectively, per ml of culture. This was achieved at 5 $\times$ 10^{-6} M TdR and 5 $\times$ 10^{-5} M inosine. At all lower nucleoside concentrations studied, maximal incorporation was not attained; however, more than 60% of the nucleoside available was utilized.

The above findings indicate the need for more cautious interpretation of previously reported work on the prevention of MTX toxicity by TdR and purines in cultured cells, including S-180 sarcoma cells (10), L-cells (11), and L1210 cells (6,12). The minimal concentration of TdR and purines required to achieve, through their reutilization, normal thymine and purine nucleotide synthesis during a block in the de novo synthetic pathways appear to be less than was

previously thought. L1210 cells are capable of efficient salvage of TdR and purines at concentrations of 10^{-6} M or less.

PRPP and Cellular Purine Salvage Capacity

Changes in cellular PRPP content during exposure of L1210 cells to MTX in vitro have been examined (17). PRPP levels fluctuate in untreated cells during logarithmic growth with a mean of 145 ± 65 (SD) pmol/10^6 cells. Treatment with MTX, 10^{-8} to 10^{-6} M, results in increased PRPP levels as high as 1,000 pmol/10^6 cells. MTX concentrations of 10^{-7} and 10^{-6} M yield rapid increases within 1.5 hours, while at the lower concentration, 10^{-8} M, increased PRPP levels are not evident until 6 hours after MTX treatment. During MTX exposure PRPP levels remain elevated for 12 to 24 hours. The effect of MTX on cellular PRPP content appears to be due to the inhibitory effect of MTX on de novo purine synthesis, resulting in an accumulation of PRPP. This effect does not involve an increase in PRPP synthetase activity. At least a portion of the increased amounts of PRPP in MTX-treated cells is used for de novo uracil nucleotide synthesis, as evidenced by an increase in cellular UTP levels (18). We have shown in a previous study that these enhanced PRPP levels are markedly decreased after the addition of hypoxanthine, indicating their utilization in purine salvage (17).

TABLE 1.—Utilization of ^{14}C-thymidine and ^{14}C-inosine from medium by L1210 cells in vitro after 24 hours*

Initial concentration mol/l	Distribution of ^{14}C-nucleoside (1 ml culture)		
	Total nmol	Nucleic acids nmol	Medium nmol (% initial)
	^{14}C-thymidine		
5×10^{-7}	0.5	0.36	0.06 (12%)
1×10^{-6}	1	0.71	0.12 (12%)
5×10^{-6}	5	2.0	2.0 (40%)
1×10^{-5}	10	2.0	6.3 (63%)
	^{14}C-inosine		
1×10^{-6}	1	0.78	0.08 (8%)
5×10^{-6}	5	3.90	0.34 (7%)
1×10^{-5}	10	4.60	0.86 (9%)
5×10^{-5}	50	9.20	27.40 (55%)
1×10^{-4}	100	8.20	73.80 (74%)

*Cultures at an initial cell concentration of 2×10^5 cells/ml were incubated for 24 hours with either ^{14}C-methyl-TdR (46.6 mCi/mmol) or 8-^{14}C-inosine (62 mCi/mmol). Cultures tested for ^{14}C-TdR utilization were supplemented with 10^{-4} M inosine and those tested for ^{14}C-inosine utilization were tested with 10^{-5} M TdR. At the end of incubation radioactivity was measured in the cell-free medium and in the trichloroacetic acid-precipitated material of cells. The data represent averages of values (less than 15% variation) from duplicate cultures. Counts in nucleic acids were determined from the trichloroacetic acid precipitate.

TABLE 2.—Utilization of ^{14}C-thymidine and ^{14}C-inosine from medium by L1210 cells in vitro after 24 hours in the presence of MTX*

Initial concentration mol/liter	Distribution of ^{14}C-nucleoside (1 ml culture)		
	Total nmol	Nucleic acids nmol	Medium nmol (% initial)
	^{14}C-thymidine		
5×10^{-7}	0.5	0.46	0.08 (16%)
1×10^{-6}	1	0.62	0.10 (10%)
5×10^{-6}	5	2.0	2.1 (42%)
1×10^{-5}	10	1.9	6.9 (69%)
	^{14}C-inosine		
1×10^{-6}	1	0.64	0.19 (19%)
5×10^{-6}	5	3.4	0.83 (17%)
1×10^{-5}	10	6.9	1.4 (60%)
5×10^{-5}	50	10.2	30.2 (60%)
1×10^{-4}	100	10.6	75.8 (76%)

*Conditions and methods were the same as described in table 1 except that 10^{-7} M MTX was present in all cultures during incubation. Data represent averages of values (less than 15% variation) from duplicate cultures.

To determine if changes in PRPP cellular levels affect the purine salvage capacity of L1210 cells, we studied the incorporation of ^{14}C-hypoxanthine into nucleotides and nucleic acids in cells treated with MTX. Table 3 shows the comparison of hypoxanthine utilization by untreated control cells and MTX-treated cells. At concentrations of 5×10^{-6} and 10^{-5}M, hypoxanthine was incorporated into either fraction twice as much in the MTX-treated cells. At 10^{-6} M hypoxanthine, incorporation into the nucleic acid fraction was increased less than twofold. Total incorporation of ^{14}C-hypoxanthine was doubled in MTX-treated cells at 5×10^{-6} and 10^{-5} M hypoxanthine, but not at 10^{-6} M hypoxanthine. At 5×10^{-6} M, total incorporation is near maximal at 0.7 and 1.5 nmol/10^{-6} cells for untreated and MTX-treated cells, respectively. These values are higher than the PRPP values observed and suggest that the rate of PRPP synthesis may also be an important factor in the salvage of hypoxanthine. In our earlier study we noted that the depletion of PRPP levels in L1210 cells with hypoxanthine (10^{-4} M) was reversed after the removal of hypoxanthine following 1.5 hours of MTX exposure. This recovery of PRPP levels was much less when examined 10 hours after MTX. Prolonged exposure of cells to MTX appears to diminish the cell's capacity to synthesize PRPP. The maintenance or recovery of adequate synthesis of PRPP can be a critical determinant in purine protection or rescue from MTX cytotoxicity.

TABLE 3.—Enhanced ^{14}C-hypoxanthine utilization in MTX-treated L1210 cells in vitro*

^{14}C hypoxanthine concentration in medium (mol/liter)	Control (nmol/10^6 cells)		MTX-treated (nmol/10^6 cells)	
	Nucleotides	Nucleic acids	Nucleotides	Nucleic acids
1×10^{-6}	0.21	0.13	0.16	0.23
5×10^{-6}	0.43	0.26	1.01	0.51
1×10^{-5}	0.48	0.27	1.25	0.58

*Cells in logarithmic growth were placed in fresh medium at a concentration of 10^6 cells/ml in the absence and presence of 10^{-7} M MTX and incubated at 37 °C for 3 hours. Cells were subsequently transferred to fresh medium again at a concentration of 10^6 cells/ml. 8-^{14}C-hypoxanthine (52 mCi/mmol) was added to the cultures at different concentrations and the cells were incubated at 37 °C for 20 minutes. Radioactivity was measured in the trichloroacetic acid-precipitated material (nucleic acids) and in the fraction of the acid soluble portion that was adsorbed to DEAE-cellulose (nucleotides). Counts per minute were converted to nmol. The data represent averages of values (less that 10% variation) from duplicate cultures.

REFERENCES

1. BERTINO JR. "Rescue" techniques in cancer chemotherapy: use of leucovorin and other rescue agents after methotrexate treatment. Semin Oncol 4:203–216, 1977.
2. ENSMINGER WD, and FREI E. The prevention of methotrexate toxicity by thymidine infusion in humans. Cancer Res 37:1857–1865, 1977.
3. HOWELL SB, ENSMINGER WD, KRISHAN A, ET AL. Thymidine rescue of high dose methotrexate in humans. Cancer Res 38:325–330, 1978.
4. SCHORNAGEL JH, LEYVA A, BUESA JM, ET AL. Thymidine prevention of methotrexate toxicity in head-and-neck cancer. *In* Clinical Pharmacology of Anti-Neoplastic Drugs (Pinedo HM, ed). Amsterdam, Elsevier/North Holland Biomedical Press, 1978, pp 83–96.
5. PINEDO HM, ZAHARKO DS, BULL JM, ET AL. The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. Cancer Res 36:4418–4424, 1976.
6. LEYVA A, VAN DER GRINT L, and PINEDO HM. Reversal of methotrexate toxicity to mouse bone marrow and L1210 leukemia cells grown in vitro. *In* Clinical Pharmacology of Anti-Neoplastic Drugs (Pinedo HM, ed). Amsterdam Elsevier/North Holland Biomedical Press, 1978, pp 39–56.
7. NAHAS A, NIXON P, and BERTINO JR. Uptake and metabolism of ^5N-formyl tetrahydrofolate by L1210 leukemia cells. Cancer Res 32:1416–1421, 1972.
8. GOLDIN A, VENDETTI JM, KLINE I, ET AL. Eradication of leukaemic cells (L1210) by methotrexate and methotrexate plus citrovorum factor. Nature 212:1548–1550, 1960.
9. SIROTNAK FM, MOCCIO DM, and DORICK DM. Optimization of high dose methotrexate with leucovorin rescue therapy in the L1210 leukemia and sarcoma 180 murine tumor models. Cancer Res 38:345–352, 1978.
10. HAKALA MT, and TAYLOR E. The ability of purine and thymine derivatives and of glycine to support the growth of mammalian cells in culture. J Biol Chem 234:126–128, 1959.
11. BORSA J, and WHITMORE GF. Studies relating to the mode of action of methotrexate. II. Studies on sites of action in L-cells in vitro. Mol Pharmacol 5:303–317, 1969.
12. GRINDEY GB, and MORAN RG. Effects of allopurinol on the therapeutic efficacy of methotrexate. Cancer Res 35:1702–1705, 1975.
13. TATTERSALL MHN, JACKSON RC, JACKSON STM, ET AL. Factors determining cell sensitivity to methotrexate: studies of folate and deoxyribonucleoside triphosphate pools in five mammalian cell lines. Eur J Cancer 10:819–826, 1974.
14. JACOBS SA, STOLLER RG, CHABNER BA, ET AL. 7-hydroxymethotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high dose methotrexate. J Clin Invest 57:534–538, 1976.
15. LANKELMA J, VAN DER KLEIJN E, and TERMOND EFS. Assay of methotrexate and 7-hydroxymethotrexate by high pressure liquid chromatography and its application to clinical pharmacokinetics. *In* Clinical Pharmacology of Anti-Neoplastic Drugs (Pinedo HM, ed). Amsterdam, Elsevier/North Holland Biomedical Press, 1978, pp 13–28.
16. GOLDMAN ID. Effects of methotrexate on cellular metabolism: some critical elements in drug-cell interaction. Cancer Treat Rep 61:549–556, 1977.
17. BUESA-PEREZ JM, LEYVA A, and PINEDA HM. Effect of methotrexate on 5-phosphoribosyl 1-pyrophosphate levels in L1210 leukemia cells in vitro. Cancer Res 40:139–144, 1980.
18. HRYNIUK WM, BROX LW, HENDERSON JF, ET AL. Consequences of methotrexate inhibition of purine biosynthesis in L1578Y cells. Cancer Res 35:1427–1432, 1975.

Clinical Pharmacology of Methotrexate [1]

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While pharmacologic considerations have enhanced the use of many antineoplastic drugs, none of these drugs has been studied as thoroughly or monitored in routine use as carefully as methotrexate (MTX). This intense interest in the clinical pharmacology of MTX has largely stemmed from the use of high-dose methotrexate (HDMTX) infusions in osteosarcoma, with its attendant 10% incidence of severe myelosuppression, mucositis, and occasional fatalities (1). The need for prediction and prevention of this toxicity was a major factor in spurring the development of suitable assay procedures and the careful exposition of clinical pharmacokinetics. This paper will review the current status of MTX clinical pharmacology, with particular emphasis on recent progress in understanding the causes of drug toxicity and in averting such toxicity by routine monitoring.

ANIMAL STUDIES

Zaharko et al developed a detailed model for MTX pharmacokinetics that accurately predicted drug-derived radioactivity in various tissue compartments for a 4-hour period following drug administration (2). The main features of this model were quantitative elimination by renal excretion, an active enterohepatic recirculation of drug, and tissue absorption dependent on blood flow into individual compartments. Multiple half-lives were detected in plasma, the longest and most important being 3 hours in duration. A similar but more limited study of ^{3}H-MTX disposition in man confirmed similar characteristics of drug distribution and pharmacokinetics, but also detected a late half-life of 27 hours (3). However, both these studies lacked a specific assay and did not differentiate between parent drug and inactive metabolites. The use of more specific assay methods has considerably altered this initial conception of MTX pharmacokinetics.

[1] The authors would like to acknowledge the many contributions of their colleagues, Drs. Charles Myers, Kenneth Hande, Ronald Stoller, and Samuel Jacobs, to our current understanding of this subject. We would also like to thank Kathleen Moore for her very efficient editorial assistance.

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ABSORPTION

MTX is completely absorbed from the gastrointestinal tract at doses below 30 mg/m^2 (4), but at higher doses absorption is incomplete. However, the oral route is not commonly used for induction of high-dose therapy, since this type of administration subjects the drug to the vagaries of intestinal absorption, intestinal bacterial degradation, and hepatic metabolism, all of which may alter the dose that ultimately reaches the systemic circulation.

DISTRIBUTION

MTX distributes in total body water; intracellular penetration depends on an active transport into cells, as reviewed elsewhere in this symposium. The drug is loosely bound to serum albumin, with approximately 40% binding at 1 μM concentrations in plasma; however, there is little evidence that this binding is of any significance in determining drug action or interactions. The drug penetrates slowly into third space collections such as pleural fluid or ascites, reaching equilibrium with plasma concentrations in approximately 6 hours. Thereafter, the slow clearance from third spaces, in comparison to clearance from the systemic circulation, produces a concentration gradient of several-fold in favor of the isolated compartment. Clearance from peritoneal fluid is about 5 ml/minute, which is substantially less than the systemic clearance, which equals or exceeds the glomerular filtration rate (5,6). Third space retention of MTX following iv administration has been associated with a prolongation of the final half-life in plasma, since the ascites tends to act as a reservoir for slow reentry of drug back into the circulation. This effect should be kept in mind in patients with pleural or peritoneal fluid collections who are receiving HDMTX.

PLASMA PHARMACOKINETICS

Following an initial distribution phase lasting a very few minutes, drug disappearance from plasma has two components. Conventional doses of 100 mg/m^2 or less produce peak concentrations in plasma of approximately 10 μM, while high-dose infusions of 1.5 g/m^2 yield higher levels of 0.1 to 1 mM (7). The initial

disappearance phase has a half-life of 2 to 3 hours. This phase extends for a period of about 12 hours, and is largely determined by renal excretion of drug, as over 50% of an administered dose is excreted in this time period. In patients with normal creatinine clearance, the half-life of the initial phase increases with advancing age of the patient, lending variability to plasma levels, pharmacokinetics, and toxicity.

The final phase of drug disappearance has a longer half-life, approximately 10 hours, but this may be affected by third space fluid or other, less understood factors. This terminal phase begins at drug concentrations above the threshold for toxicity to bone marrow (1×10^{-8} M) or gastrointestinal epithelium (5×10^{-9} M) (8). A prolongation of this terminal phase is often associated with unexpected and, at times, severe toxicity.

MTX may be administered by constant infusion; this route of administration has the advantage of producing predictable blood and cerebrospinal fluid concentrations for a specified period of time. Bleyer (9) has found that the following formula achieves a desired plasma concentration in patients with normal renal function:

Priming dose in $mg/m^2 = 1.5 \times 10^7$. [MTX] plasma;

Infusion dose in $mg/m^2/hour = 3 \times 10^6$. [MTX] plasma.

An approximate correction for reduced renal function can be made by reducing the dose in proportion to the reduction in creatinine clearance, based on a normal value of 60 ml/minute/m^2.

RENAL EXCRETION

Most of the administered drug is excreted unchanged in the urine in the first 12 hours. Estimates vary from 44% to 100% of the dose for patients with normal renal function (9,10). Renal clearance has been variously reported as greater than, equal to, or less than creatinine clearance, with some estimates as high as 194 ml/minute (5,10–12). Because of the high concentrations in the urine during HDMTX infusion, alkalinization and forced diuresis are recommended for maintaining solubility of the drug and its metabolites (vide infra).

MTX is believed to be excreted both by glomerular filtration and by tubular secretion. Probenecid, which inhibits excretion of many weak organic acids, also blocks MTX excretion and prolongs the plasma pharmacokinetic decay curve (12). In general, MTX pharmacokinetics are not entirely predictable on the basis of renal function; it is this author's suspicion that altered tubular function, not necessarily reflected in measurements of creatinine clearance, may be responsible for this variability.

HEPATIC UPTAKE AND BILIARY EXCRETION

MTX is taken up by hepatocytes by an active transport process (13,14) and, within the liver, is metabolized to at least two derivatives, 7-hydroxy MTX (15) and polyglutamates of MTX (16). The former has been identified in urine (15) and plasma (17), and may play a role in the renal precipitation that occurs in some patients during high-dose infusions; it has much less solubility than the parent drug at neutral and acid pH levels. The significance of polyglutamation is not understood at this time, although these forms persist in the liver for many months.

MTX polyglutamates are also formed in tumor cells (18) and normal skin fibroblasts (19) and, in view of their limited permeability, may persist inside the cell long after the disappearance of free parent compound. This persistence may prolong the effective duration of action of the drug in tissues capable of this transformation (19).

There is also evidence for enterohepatic recirculation of MTX, although the quantitative significance of this process has not been established in man (10,20). In the few patients with biliary fistulae studied thus far, less than 20% of an administered dose was found in the bile in specimens collected over a several-day period. Through the process of biliary excretion, methotrexate enters the gastrointestinal tract, where a fraction is metabolized to 2,4-diamino-N^{10}-methylpteroic acid (DAMPA). The latter is found in blood and urine (21) in increasing quantities with time, and cross-reacts with most antibodies used in the radioimmunoassay systems for methotrexate. Like 7-hydroxy MTX, DAMPA has limited aqueous solubility, but neither metabolite is believed to have sufficient antifolate activity to augment the antineoplastic action of MTX.

TOXICITY

Folate antagonists exert their primary toxic effects on rapidly dividing tissues, including the host bone marrow and gastrointestinal epithelium. Leukopenia and thrombocytopenia reach their nadir 7 to 10 days after drug administration; both counts recover within 7 days of reaching their nadir, although the duration of myelosuppression may be influenced by drug dose and individual pharmacokinetics, particularly by the duration of persistence of free drug concentrations in the peripheral circulation. In order to detect delayed elimination and predict a high risk of myelosuppression, plasma MTX measurement has been employed in selected situations.

In most clinical circumstances, day-to-day monitoring of MTX blood levels is unnecessary, since pretreatment assessment of renal function provides an approximate guide as to the likelihood of handling this drug in a normal way. However, with HDMTX regimens, the ability to excrete this agent is not

always predictable on the basis of pretreatment renal evaluation. These regimens, which employ otherwise lethal doses in 6- to 42-hour infusions, followed by a 24- to 48-hour period of leucovorin (LV) rescue, depend for their safety on rapid excretion of drug and a favorable ratio between LV and MTX during the rescue period. In the few carefully studied variations of this regimen, routine monitoring was able to detect abnormally elevated drug levels at 24 or 48 hours after the initiation of drug infusion (11,22). For example, after infusion of 50 to 250 mg/kg over a 6-hour period, Stoller et al (22) found that a 48-hour plasma level of greater than 9×10^{-7} M indicated a high probability of myelosuppression. These authors also found that the institution of higher doses of LV at 48 hours (doses of 100 mg/m^2) effectively prevented leukopenia, despite the presence of high concentrations of MTX. It now appears that the LV dose can be adjusted to plasma MTX concentrations at an earlier time (23,24), although the relative safety of such "fine-tuning" as compared to blanket coverage as prescribed in most high-dose regimens has not been proven in an actual clinical trial.

Delayed drug excretion during high-dose therapy appears to result from acute toxic effects of MTX on renal function, leading to delayed drug clearance, ineffective rescue, and severe myelosuppression. Renal dysfunction is believed to result from drug precipitation in the renal tubules, as demonstrated in the monkey by Jacobs et al (15). Both known metabolites of MTX, 7-hydroxy MTX and DAMPA, have much less solubility in acid urine than does the parent compound, but neither constitutes an important fraction of excreted drug overall; these metabolites become a significant percentage of plasma and urinary material in the latter stages of drug elimination (more than 12 hours after the infusion is begun) (15,21). In view of the likelihood that urinary MTX will exceed its solubility at normal rates of urine flow, and at acid pH levels, most centers now employ vigorous hydration (3 liters/m^2/24 hours) and intravenous bicarbonate to alkalinize the urine, beginning 6 to 12 hours before the MTX infusion and continuing for 24 to 36 hours. Urine flow and pH are monitored prior to and during drug infusion to ascertain that pH is above 7.0 and urine flow exceeds 100 ml/hour. With this type of regimen, the incidence of renal failure during HDMTX administration has declined from 10% to less than 1% at the National Cancer Institute, and other institutions have reported similar favorable experience (25). Hydration does not accelerate MTX disappearance from plasma, and thus should have no deleterious effect on the therapeutic benefits of this regimen (26).

The management of patients with abnormally elevated MTX concentrations, in addition to increased doses of LV, would ideally include measures to accelerate elimination of the drug, particularly since many of these patients have abnormal renal function. Peritoneal dialysis has proven to be totally ineffective in removing drug, providing a clearance of 5 ml/minute, as compared to normal renal clearance, which exceeds 120 ml/minute. Hemodialysis is somewhat more effective (5), since clearances by this manipulation reach 35 to 40 ml/minute. However, because of reentry of drug into the plasma compartment from deeper tissue compartments, improvement in blood levels is observed only during dialysis, with rapid rebound of drug concentrations to predialysis levels once the procedure is stopped. Charcoal hemoperfusion columns encounter the same reentry problem (27), but in addition platelet consumption by these columns may lead to thrombocytopenia. Other workers have demonstrated the ability of a bacterial enzyme, carboxypeptidase G_1 to cleave circulating methotrexate, and to rescue patients receiving HDMTX (28). This enzyme is not commercially available, but may well be the ideal approach to the problem of drug elimination in the patient with renal failure.

In addition to its effects on rapidly dividing tissues, toxicity to liver and lung and, occasionally, anaphylaxis have been reported. Chronic MTX therapy in patients with psoriasis (29), or, less commonly, in children with leukemia, may lead to portal fibrosis and frank cirrhosis. The incidence of cirrhosis in patients with psoriasis may reach 25%, but underlying chronic hepatic disease in these patients has made an accurate estimation of the drug-related toxicity difficult.

Acute elevations of SGOT, SGPT, and LDH are often seen in the week following high-dose infusions, but have not been associated with long-term sequelae, and usually rapidly normalize in a few days. The biochemical basis for hepatic toxicity, either acute or chronic, is poorly understood, although animal experiments suggest that choline may prevent chronic toxicity in rats (30).

Sporadic case reports of a self-limited pneumonitis secondary to MTX have appeared in the literature (31). Lung biopsies have revealed a variety of findings, including simple interstitial edema, a mononuclear infiltrate, and noncaseating granulomas. The reaction does not appear to be a hypersensitivity manifestation, since reinstitution of the drug has been possible in many cases. Many such patients with "MTX pneumonitis" require lung biopsy to rule out the possibility of opportunistic infection or malignancy. In the absence of a specific finding on biopsy, specific therapy is not indicated, other than withholding of MTX during the acute episode.

Actual anaphylactic reactions to MTX are rare, although recent case reports (32) described patients with acute cardiovascular collapse or acute facial edema, rash, and generalized pruritis, both of which reactions were reproduced on rechallenge with the drug. Since both patients were also receiving BCG at the time, they may have developed increased sensitivity to the drug as the result of this concomitant immunostimulation.

REFERENCES

1. VonHoff DD, Penta JS, Helman LJ, et al. Incidence of drug-related deaths secondary to high-dose methotrexate and citrovorum factor administration. Cancer Treat Rep 61:745–748, 1977.
2. Zaharko DS, Dedrick RL, Bischoff KB, et al. Methotrexate tissue distribution; prediction by a mathematical model. J Natl Cancer Inst 46:775–784, 1971.
3. Huffman DH, Wan SH, Azarnoff DL, et al. Pharmacokinetics of methotrexate. Clin Pharmacol Ther 14:572–579, 1973.
4. Henderson ES, Adamson RH, and Oliverio VT. The metabolic fate of tritiated methotrexate. II. Absorption and excretion in man. Cancer Res 25:1018–1024, 1965.
5. Hande KR, Balow JE, Drake JC, et al. Methotrexate and hemodialysis. Ann Intern Med 87:495–496, 1977.
6. Monjanel S, Rigault JP, Cano JP, et al. High-dose methotrexate: preliminary evaluation of a pharmacokinetic approach. Eur J Cancer. In press.
7. Stoller RG, Jacobs SA, Drake JC, et al. Pharmacokinetics of high-dose methotrexate. Cancer Chemother Rep 6:19–24, 1975.
8. Chabner BA, and Young RC. Threshold methotrexate concentrations for in vivo inhibition of DNA synthesis in normal and tumorous target tissues. J Clin Invest 52:1804–1811, 1973.
9. Bleyer WA. The clinical pharmacology of methotrexate. Cancer 41:36–51, 1978.
10. Calvert AH, Bondy PK, and Harrap KK. Some observations on the human pharmacology of methotrexate. Cancer Treat Rep 61:1647–1656, 1977.
11. Isacoff WH, Morrison PF, Aroesty J, et al. Pharmacokinetics of high-dose methotrexate with citrovorum factor rescue. Cancer Treat Rep 61:1665–1674, 1977.
12. Liegler DG, Henderson ES, Hahn MA, et al. The effect of organic acids on renal clearance of methotrexate in man. Clin Pharmacol Ther 10:849–857, 1969.
13. Gewirtz DA, White JC, and Goldman ID. Transport, binding and polyglutamation of methotrexate (MTX) in freshly isolated hepatocytes. Proc Am Assoc Cancer Res 20:147, 1979.
14. Strum WB, and Liem HH. Hepatic uptake, intracellular protein binding and biliary excretion of amethopterin. Biochem Pharmacol 26:1235–1240, 1977.
15. Jacobs SA, Stoller RG, Chabner BA, et al. 7-hydroxy methotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high-dose methotrexate. J Clin Invest 57:534–538, 1976.
16. Jacobs SA, Derr CJ, and Johns DG. Accumulation of methotrexate diglutamate in human liver during methotrexate therapy. Biochem Pharmacol 26:2310–2313, 1977.
17. Watson E, Cohen JL, and Chan KK. High-pressure liquid chromatographic determination of methotrexate and its major metabolite, 7-hydroxy methotrexate, in human plasma. Cancer Treat Rep 62:381–387, 1978.
18. Whitehead VM. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. Cancer Res 37:408–412, 1977.
19. Rosenblatt DS, Whitehead VM, Dupont MM, et al. Synthesis of methotrexate polyglutamates in cultured human cells. Mol Pharmacol 14:210–214, 1978.
20. Shen DD, and Azarnoff DL. Clinical pharmacokinetics of methotrexate. Clin Pharmacokin 3:1–13, 1978.
21. Donehower RC, Hande KR, Drake JC, et al. Presence of 2,4-diamino N^{10} methyl pteroic acid after high-dose methotrexate. Clin Pharm Ther 26:63–72, 1979.
22. Stoller RG, Hande KR, Jacobs SA, et al. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. N Engl J Med 297:630–634, 1977.
23. Jacobs SA, and Santicky MJ. Phase I trial of high-dose methotrexate with modified citrovorum factor rescue. Cancer Treat Rep 62:397–399, 1978.
24. Stoller RG, Kaplan HG, Cummings FJ, et al. A chemical and pharmacological study of high-dose methotrexate with minimal leucovorin rescue. Cancer Res 39:908–912, 1979.
25. Nirenberg A, Mosende C, Mehte B, et al. High-dose methotrexate with citrovorum factor rescue: predictive value of serum methotrexate concentrations and corrective measures to avert toxicity. Cancer Treat Rep 61:779–783, 1977.
26. Romolo JL, Goldberg NH, Hande KR, et al. The effect of hydration on plasma methotrexate levels. Cancer Treat Rep 61:1393–1395, 1977.
27. Djerassi I, Ciesielka W, and Kim JS. Removal of methotrexate by filtration-adsorption using charcoal filters or by hemodialysis. Cancer Treat Rep 61:751–752, 1977.
28. Abelson HT, Ensminger W, Rosowsky A, et al. Comparative effects of citrovorum factor and carboxypeptidase G_1 on cerebrospinal fluid methotrexate pharmacokinetics. Cancer Treat Rep 62:1549–1552, 1978.
29. Dahl MGC, Gregory MM, and Scheuer PJ. Liver damage due to methotrexate in patients with psoriasis. Br Med J 1:625–730, 1971.
30. Tuma DJ, Barak AJ, and Sorrell MF. Interaction of methotrexate with lipotropic factors in rat liver. Biochem Pharmacol 24:1327–1331, 1975.
31. Sostman HD, Matthay RA, Putman C, et al. Methotrexate-induced pneumonitis. Medicine 55:371–388, 1976.
32. Goldberg NH, Romolo JL, Austin EH, et al. Anaphylactoid type reactions in two patients receiving high-dose intravenous methotrexate. Cancer 41:52–55, 1978.

Techniques for the Measurement of Methotrexate in Biological Samples [1]

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SUMMARY

Techniques available for the measurement of methotrexate (MTX) in biological fluids are reviewed. The importance of measuring MTX in plasma following high-dose MTX treatment is now well recognized and renewed interest in the metabolism and pharmacokinetics of the drug has prompted the development of new techniques of analysis. Results obtained with our radioimmunoassay are presented and compared with results obtained by other methods.

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Since its introduction as an antifolate drug many different techniques for the measurement of methotrexate (MTX) have been described. Much valuable information on the pharmacokinetics and metabolism of the drug has been obtained using tritiated drug (1). However, since the introduction of high-dose treatment of several diseases, and renewed interest in the pharmacokinetics of the drug, several sensitive, more specific methods suitable for large batches of samples have been developed. Methods are required for rapid monitoring of drug blood levels following high doses of MTX so that individuals with potentially toxic levels of drug may be identified and the appropriate "rescue" treatment can be instituted (2). The choice of assay depends on a number of factors, especially on the specificity and sensitivity of the technique. The ideal assay should not be affected by endogenous substances such as reduced folates, especially when these are artificially raised during folinic acid rescue. Now that there is increasing evidence of metabolism of MTX in man, especially following high doses of the drug (3,4), metabolites of MTX should also be tested for an effect on the assay. Most techniques in common use can measure levels of MTX as low as 2×10^{-8} M. The extreme sensitivity possible with some methods, in which as little as 25 pg can be measured, may be a disadvantage, since large serial dilutions of samples are necessary, depending on the dose of the drug, the time of the sample following administration, and patient variability. On the other hand, high sensitivity can be used to advantage in long-term studies on drug disappearance as well as in situations where only small sample volumes are available, eg, in repeated sampling as in pharmacokinetic work and in pediatric work.

Biological assays (5), which depend on the inhibition of the growth of microorganisms susceptible to MTX, although sensitive to 2×10^{-8} M, have poor specificity. They are also time consuming and highly variable. The fluorescence of the pteridine nucleus has been utilized (6) to measure MTX, but other pteridines may also be recorded. The sensitivity of fluorescent methods (limit of 1×10^{-7} M) is lower than for alternative techniques. Even this level may be difficult to achieve in practice.

METHODS UTILIZING DIHYDROFOLATE REDUCTASE (DHFR)

Several techniques for measuring MTX utilize the binding of the drug to the target enzyme dihydrofolate reductase (DHFR). DHFR has been obtained from lysates of liver, bacteria, yeasts, and leukemic cells. Commercial preparations are now available. While MTX has a high affinity for DHFR, any other substance that binds to the enzyme, eg, reduced folates or MTX metabolites, is theoretically able to interfere in the assay of MTX depending on its affinity for the enzyme and on its concentration. Such interference has not been shown to be significant in practice.

Chromatographic separation of the products of the enzyme reaction in the presence of MTX and radiolabelled substrate is the basis of a tedious and time-

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consuming radioenzymatic assay (7). An enzyme inhibition assay (8) has been widely used for pharmacokinetic studies and results can be obtained in a matter of minutes once the assay is set up. The decrease in enzyme activity following inhibition by MTX is measured by following the decrease in absorbance at 340 nm as NADPH is oxidized to NADP and as dihydrofolate is reduced to tetrahydrofolate. This method has a sensitivity of approximately 2×10^{-8} M, but this can be bettered if a sensitive spectrophotometer is used. A modification of this method that allows laboratories lacking sophisticated equipment and expertise in enzyme kinetics to perform the assay has been described (9).

Radioligand assays or competitive protein binding (CPB) assays have been described, with DHFR used as the binding protein. These assays are suitable for both pharmacokinetic studies and clinical monitoring, results of batches of samples being available within 1 to 2 hours. Although technically easier to perform than the DHFR inhibition assay, a radiolabelled MTX is required. In the noncompetitive system (10), radiolabelled MTX is added following the incubation of DHFR with standards or samples containing MTX, ie, to titrate unfilled enzyme binding sites. Sensitivity is as low as 1×10^{-9} M, and the procedure is rapid and simple to perform. A competitive binding assay (11) has been widely used and is the basis of a ^{125}I commercial assay kit (New England Enzyme Center, Boston, MA). Both radiolabelled MTX and MTX in standards or samples compete for DHFR binding sites. Separation of enzyme-bound and free-labelled MTX is achieved using a coated charcoal suspension. Samples containing MTX at a concentration of 1.5×10^{-9} M can be measured, ie, equivalent to a sensitivity of 25 pg MTX.

High Pressure Liquid Chromatography (HPLC)

Complete specificity for MTX can be achieved by chromatographic separation of the drug from its metabolites using HPLC, although considerable expertise is required. Techniques for measuring the drug in biological fluids have recently been described (12,13). A sensitivity of approximately 2×10^{-8} M can be achieved with the advantage that metabolites such as 7-hydroxy MTX can also be quantitated. Extraction procedures are required and the assay of one sample may take 20 to 30 minutes, so the technique does not lend itself to large batch numbers. HPLC is nevertheless becoming a very useful tool, especially for metabolic studies, although the specialized equipment and knowledge necessary may prevent its widespread use in clinical laboratories at present.

Immunoassay Techniques

Several immunoassay techniques for MTX measurement have been reported (14–16). They offer a fast, reliable, extremely sensitive method for measuring the drug and are especially useful when large batches of samples are involved. Immunoassays can be easily adopted by many clinical laboratories so that routine blood monitoring is feasible. The disadvantage of high sensitivity and a narrow range of standards is that high serial dilutions are necessary for most samples and, in may cases, repeat assays have to be carried out.

Unlike different batches of DHFR, each preparation of MTX antiserum will have different specificity characteristics, even though the antisera have been raised against similar drug-protein conjugates (usually a simple carbodiimide condensation of the glutamyl carboxyl groups of MTX with amino groups on the carrier proteins). The specificity of each immunoassay should be clearly defined, especially as far as endogenous folates, folinic acid, and MTX metabolites are concerned.

Radioimmunoassays (RIA) depend on the competition of radiolabelled and unlabelled compounds (ie, MTX) for a limited number of antibody binding sites. Tritiated MTX has been used as the labelled moiety but an alternative to the costly and time-consuming process of scintillation counting is the use of ^{125}I-labels. These can be prepared in the laboratory (17, 18), and their use shortens the time taken to complete an assay so that results obtained with RIA can be available within 1 to 2 hours if necessary. Disadvantages of the use of ^{125}I-labels are that they are a potential health hazard, and that they have a short half-life. ^{125}I-labels have also been used in commercial RIA kits (Diagnostic Biochemistry, Inc., San Diego, CA). An enzyme immunoassay (EIA) (19) has been described in which radioactive-labelled MTX is replaced by an enzyme-MTX conjugate that is stable for long periods. The assay can be performed using a simple spectrophotometer.

The RIA Developed at the University of Surrey—RIA(S)

This assay (20) utilizes an antiserum raised in a sheep against an MTX-ovalbumin conjugate. The specificity of the antiserum is shown in table 1. Because of the low cross-reactivity of naturally occurring folates, as well as folinic acid, the assay is suitable for use when these are artifically raised. The cross-reactivity shown by aminopterin is unimportant since this drug is not coadministered with MTX. It is thought that the small amounts often present as a contaminant of MTX preparations would not have a significant effect on the RIA. Three metabolites of MTX have been examined for cross-reaction and only 2,4-diamino-N^{10}-methylpteroic acid (AMPA) is significant. To test the effect of this metabolite on the assay of MTX, a serum sample pool with a known concentration of MTX was spiked with increasing amounts of AMPA (table 2). Up to an MTX:AMPA ratio of 1:1 the added AMPA did not affect the amount of MTX measured. However, if AMPA was present at a much higher ratio, eg, as might occur in urine at late time points, an overestimation of urinary MTX output

could occur. The specificity of MTS antisera with respect to these metabolites has not always been reported, but is obviously important in interpreting data.

TABLE 1.—Specificity of MTX antiserum used in RIA(S)

	Amount required for 50% inhibition (ng)	% cross-reactivity
MTX	0.65	100.00
2,4-diamino-N^{10}-methyl pteroic acid	2.10	21.00
Aminopterin	3.40	19.10
7-hydroxymethotrexate	270.00	0.23
2,4-diamino-6-methylpteridine	560.00	0.12
N^{10}-methylfolic acid	2,250.00	0.03
Folic acid, DHFA, THFA, N^5-methyl folic acid, Folinic acid, p-Aminobenzoic acid, Glutamic acid	>10,000.0	<0.0065

TABLE 2.—Effect of 2,4-diamino-N^{10}-methyl pteroic acid (AMPA) on the measurement of MTX in serum

	Measured MTX concentration (ng/ml)
Pooled serum sample without added AMPA	116
Pooled serum sample + 10 ng AMPA	112
Pooled serum sample + 20 ng AMPA	94
Pooled serum sample + 50 ng AMPA	107
Pooled serum sample + 100 ng AMPA	111

The lower detection limit of RIA(S) is 1 nM, which is equivalent to aproximately 1 ng/ml in the sample. Known amounts of MTX added to serum urine and CSF can be quantitatively estimated. Intra-assay variation is 2%, and interassay variation depended on drug concentration: 5.8% at a concentration of 105 ng/ml (n=10) or 9.3% at a concentration of 1,019 ng/ml (n=6). This assay has been in regular use for 4 years, and during this time the label has been changed for practical convenience from tritiated MTX to a ^{75}Se-MTX ligand (21) and finally to a ^{125}I-MTX label (18) without any significant change in assay performance.

The EMIT® kit for measuring MTX (Syva, Palo Alto, CA) is a homogeneous immunoassay technique (22) useful for monitoring drug blood levels in patients treated with potentially toxic doses of MTX. This method is rapid and simple to perform and, providing suitable spectrophotometers are available, within the scope of most clinical laboratories. This method utilizes the reduction of enzyme activity that ensues when an enzyme-drug conjugate (in this case glucose-6-phosphate dehydrogenase) is incubated with antibodies to the drug. Free drug in the sample competes with the enzyme-labelled drug for antibody, thereby decreasing the antibody-induced inhibition of the enzyme. The absorbance change resulting from the enzyme's catalytic action on a substrate correlates with the concentration of free drug present.

The EMIT® MTX calibration curves run from 0.2×10^{-6} M to 2.0×10^{-6} M and the protocol allows for serial dilutions of samples to be made. Unlike some other methods of analysis, it is immediately apparent if further dilutions of sample are required. However, there is no provision for measuring MTX levels below 0.2×10^{-6} M. Although this sensitivity may be adequate for detecting toxic levels, the method cannot be used for most pharmacokinetic studies.

TABLE 3.—EMIT® precision study

	Using 1.0 μM calibrator over 8 days			
Sample number	1	2	3	4
Mean MTX concentration n = 20	1.087	1.117	1.06	1.048
Coefficient of variance %	9.7	9.3	5.9	7.3
Between run coefficient of variance = 2.9%	Mean 1.078 μM			

In an evaluation of the EMIT® assay the intra-assay variation for the 1×10^{-6} M calibrator was higher than that for a similar concentration obtained with RIA(S) (table 3), but the interassay variation of a sample pool stored at 4 °C and -20 °C compares with that obtained with RIA(S) (table 4). Limited specificity studies were carried out that indicated that 7-hydroxy-MTX, folinic acid, folic acid, trimethoprim, and triampterene did not cross-react. It is apparent from the EMIT® literature that there is considerable cross-reactivity between AMPA and the antibody.

TABLE 4.—EMIT®—Between assay precision

Sample number	A		B	
	Mean conc. μM	CV	Mean conc. μM	CV
1	0.24	14.3	0.26	8.8
2	0.67	7.6	0.64	1.8
3	13.42	14.4	12.77	8.6
4	0.58	6.5	0.54	6.5
5	6.65	9.2	6.25	0.6

A—Storage at 4 °C. Assayed on five occasions over 10 days.
B—Storage at -20 °C. Assayed on three occasions over 6 weeks.

COMPARISON OF TECHNIQUES

Because several methods for measuring MTX are now in use, it is important that comparisons of results obtained from different methods be made. Close agreement has been reported between the CPB assay and the DHFR inhibition assay (11), between RIA and DHFR inhibition assay (15), between RIA and CPB (23), a fluorimetric assay and RIA (24), and between

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CPB and HPLC (13), although the latter was found to be more specific.

In comparison of the RIA(S) and an RIA developed at St. Bartholomew's Hospital [RIA(K)], a close correlation of results was obtained ($r = 0.954$; $y = 5.1 + 0.982x$) (fig 1). Similar correlations have been obtained between RIA(S) and other RIA methods, in spite of the fact that different antibody preparations and different types of labels were used in the assays.

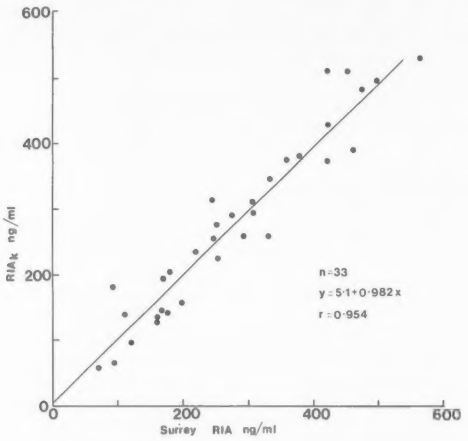

FIGURE 1.—Comparison of results obtained from two radioimmunoassays.

A set of serum samples from patients receiving high-dose MTX were assayed using the EMIT®-MTX kit in two centers (Southampton General Hospital by Dr. A. Arnold, and at the University of Surrey). Samples were assayed at the same time following storage at -20 °C (fig 2). A close correlation existed between the results obtained at the two centers ($n = 34$, $r = 0.976$, $\log y = -0.02 + 0.994 \log x$). The logarithmic figures were used because of the wide concentration range (0.2×10^{-6} M to 50×10^{-6} M). The mean value for EMIT® (S) was 2.1×10^{-6} M and for EMIT® (SGH) 2.0×10^{-6} M. Comparison of the results obtained using RIA(S) and EMIT® (S) was also good; ($n = 41$, $r = 0.974$, $\log y = -0.13 + 1.097 \log x$) (fig 3), although the EMIT® results consistently measured a higher concentration (1.54×10^{-6} M) than RIA(S) (1.2×10^{-6} M).

Serial plasma samples obtained following an iv bolus of 1,250 mg of MTX were assayed using the DHFR inhibition assay (8) by Dr. A. H. Calvert and by RIA(S). Samples were assayed several months apart (fig 4), and although there was no significant difference between the results, the RIA measured higher levels than the enzyme assay and the results varied by an almost constant factor. Pharmacokinetic interpretation of both sets of results would differ very

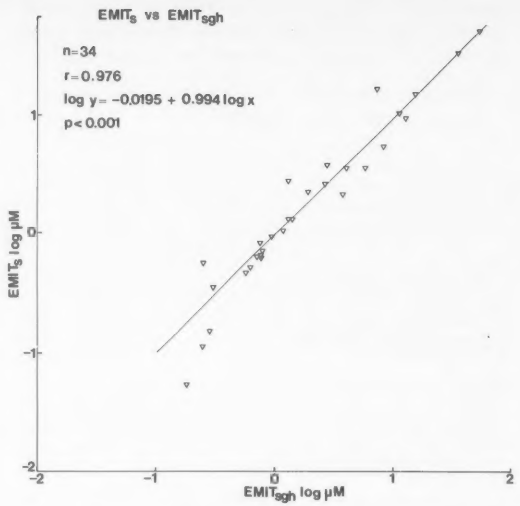

FIGURE 2.—Comparison of results obtained with EMIT®-MTX kit at two centers. Serum samples obtained following high-dose MTX.

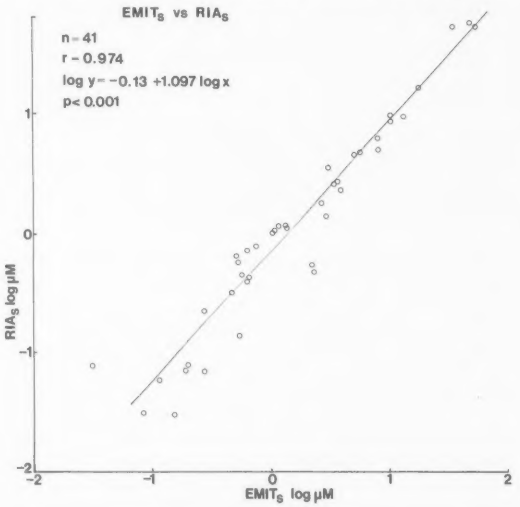

FIGURE 3.—Comparison of results obtained with a radioimmunoassay and the EMIT®-MTX kit. Samples obtained following high-dose MTX.

little, and clinical assessment of potential toxicity would undoubtedly be the same. Urine samples from the same patient were also analyzed and the results differed markedly, so that after 4 hours results obtained by RIA became progressively higher than those obtained by DHFR inhibition. These facts may reflect the effect of AMPA or other MTX breakdown products on the RIA.

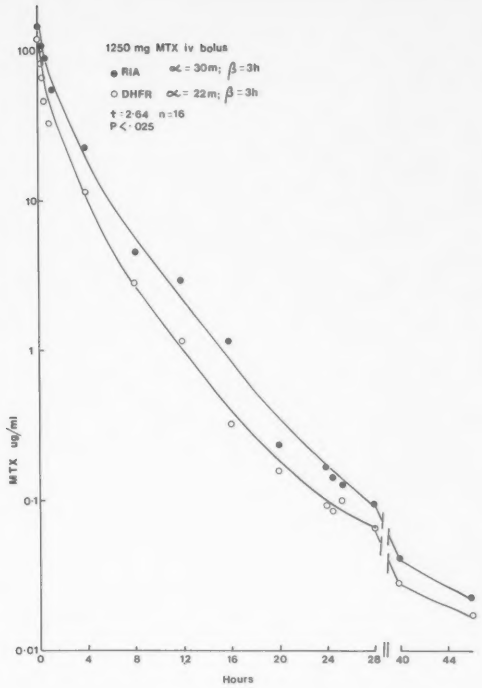

FIGURE 4.—Comparison of results obtained by DHFR inhibition assay and by RIA on plasma samples obtained following an iv bolus of 1,250 mg MTX.

CONCLUSIONS

It is apparent that there can be a considerable difference in results obtained using different techniques for measuring MTX. These differences may be statistically significant. As more information on blood drug levels is collected at different centers, it is increasingly important to be aware of possible discrepancies in reported levels. Consequently, there is a need for a properly organized exchange of standards and samples on a wide scale.

The choice of technique for MTX measurement adopted by a particular laboratory will depend on the available facilities and experience, as well as on the purpose of the study. The EMIT® system is suited to routine monitoring in the clinic, but for batch analysis of samples from both clinical trials and animal studies, RIA, CPB, or enzyme inhibition methods are preferable, whereas detailed metabolic studies would benefit from the use of an HPLC system.

REFERENCES

1. HENDERSON ES, ADAMSON RH, and DENHAM C. The metabolic fate of tritiated methotrexate. II. Absorption and excretion in man. Cancer Res 25:1018–1024, 1965.
2. STOLLER RG, HANDE KR, JACOBS SA, ET AL. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. N Engl J Med 297:630–634, 1977.
3. JACOBS SA, STOLLER RG, CHABNER BA, ET AL. 7-hydroxymethotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high dose methotrexate. J Clin Invest 57:534–538, 1976.
4. DONEHOWER RC, HANDE KR, DRAKE JC, ET AL. Presence of 2.4-diamino-N^{10}-methyl pteroic acid after high-dose methotrexate. Clin Pharmacol Ther 26:63–72, 1979.
5. BURCHENAL JH, WARING GB, ELLISON RR, ET AL. A simple method for determination of levels of amethopterin in blood and urine. Proc Soc Exp Biol Med 78:603–606, 1951.
6. FREEMAN MV. A fluorimetric method for the measurement of 4-amino-10-methyl pteroyl glutamic acid (Amethopterin) in plasma. J Pharmacol Exp Ther 120:1–8, 1957.
7. ROTHENBERG SPA. A radioenzymatic assay for folic acid antagonist in biological fluids. J Lab Clin Med 66:294–303, 1965.
8. BERTINO JR, and FISCHER FA. VI. Techniques for study of resistance to folic acid antagonists. Methods Med Res 10:297–307, 1964.
9. FALK LC, CLARK DR, KALMAN SM, ET AL. Enzymatic assay for methotrexate in serum and cerebrospinal fluid. Clin Chem 22:785–788, 1976.
10. ARONS E, ROTHENBERG SP, DA COSTA M, ET AL. A direct ligand-binding radioassay for the measurement of methotrexate in tissues and biological fluids. Cancer Res 35:2033–2038, 1975.
11. MYERS CE, LIPPMAN ME, ELIOT HM, ET AL. Competitive protein binding assay for methotrexate. Proc Natl Acad Sci USA 72:3683–3686, 1975.
12. LANKELMA J, and POPPE H. Determination of methotrexate in plasma by on-column concentration and ion-exchange chromatography. J Chromatogr 149:587–598, 1978.
13. WATSON E, COHEN JL, and CHAN KK. High pressure liquid chromatographic determination of methotrexate and its major metabolite, 7-hydroxymethotrexate, in human plasma. Cancer Treat Rep 62:381–387, 1978.
14. BOHUON C, DUBREY F, and BOUDENE C. Radioimmunoassay of methotrexate in biological fluids. Clin Chim Acta 57:263–267, 1974.
15. RASO V, and SCHREIBER R. A rapid and specific radioimmunoassay for methotrexate. Cancer Res 35:1407–1410, 1975.
16. HENDEL J, SAREK LJ, and HVIDBERG EF. Rapid radioimmunoassay for methotrexate in biological fluids. Clin Chem 22:813–816, 1976.
17. PAXTON JW, ROWELL FJ, and CREE GM. Comparison of three radioligands, selenium-75, iodine-125, and tritium, in the radioimmunoassay for methotrexate. Clin Chem 24:1534–1538, 1978.
18. KAMEL RS, and GARDNER J. Preparation of 125-iodine/labelled methotrexate and its use in a magnetisable particle solid-phase radioimmunoassay. Clin Chim Acta 89:363–370, 1978.
19. AL-BASSAM MN, O'SULLIVAN MJ, BRIDGES JW, ET AL. Improved double-antibody enzyme immunoassay for methotrexate. Clin Chem 25:1448–1452, 1979.
20. AHERNE GW, PIALL EM, and MARKS V. Development and application of a radioimmunoassay for methotrexate. Br J Cancer 36:608–617, 1977.
21. AHERNE W, PIALL E, and MARKS V. Radioimmunoassay of methotrexate: use of ^{75}Se-labelled methotrexate. Ann Clin Biochem 15:331–334, 1978.

22. Rubenstein KE, Schneider RS, and Ullman EF. Homogeneous enzyme immunoassay—a new immunochemical technique. Biochem Biophys Res Comm 47:846–851, 1972.
23. Virtanen R, Iisalo E, Parvinen M, et al. Methotrexate concentrations in biological fluids: comparison of results obtained by radioimmunoassay and direct ligand binding radioassay. Acta Pharmacol Toxicol 44:296–302, 1979.
24. Loeffler LJ, Blum MR, and Nelson MA. A radioimmunoassay for methotrexate and its comparison with spectrofluorimetric procedures. Cancer Res 36:3306–3311, 1976.

Adjuvant Combination Chemotherapy for Operable Breast Cancer. Trials in Progress at the Istituto Nazionale Tumori of Milan[1]

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SUMMARY

Adjuvant trials ongoing at the Istituto Nazionale Tumori of Milan for operable breast cancer with nodal involvement are reviewed in this paper. In the first trial (cyclophosphamide, methotrexate, 5-fluorouracil [CMF] versus control), the early 5-year results confirm the usefulness of combination chemotherapy in significantly affecting the relapse-free survival (RFS) and total survival rates. The degree of axillary node involvement remains the most important prognostic indicator also in patients receiving adjuvant chemotherapy. The contention concerning the therapeutic effect of CMF in pre- versus postmenopausal women is overcome by the observation that about 80% of patients receiving >85% of the planned dose are surviving relapse free at 5 years regardless of their menopausal status. The 3-year results of the second CMF trial indicate that both RFS and total survival are comparable between the groups receiving 12 or 6 cycles of adjuvant CMF. The results were not affected by drug-induced amenorrhea nor by estrogen receptor status. Provided the percent of dose administered is high, there is probably no real advantage in prolonging CMF chemotherapy beyond the sixth month. However, the results are still preliminary.

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The initial favorable results of the first randomized trials have provided the impetus for introducing adjuvant chemotherapy into contemporary research programs for multidisciplinary treatment of primary breast cancer (1). The scientific and biological questions to be asked from the new strategy are numerous. From the clinical point of view, the most important question to be answered is, "can adjuvant chemotherapy improve the relapse-free survival (RFS) and total survival with an acceptable degree of morbidity?" Problems involved in the organization of such a long-term experiment are complex, and it is estimated that a decade of consecutive clinical trials will be required to define the clinical value of adjuvant chemotherapy. In order that facts, ie, therapeutic results, may gradually substitute for opinion, it is essential that surgeons, radiation therapists, and medical oncologists take active part in controlled clinical trials aimed at defining the value of various forms of adjuvant therapy.

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This paper provides a brief overview of prospective controlled adjuvant trials ongoing at the Istituto Nazionale Tumori of Milan. Since 1973, study design, patient accrual, treatment performance, and evaluation have been enormously facilitated by the continuous cooperation of clinical staff who have accepted the concept that multidisciplinary therapy may offer promise for improving the cure rates of common cancers.

ADJUVANT STUDIES

Table 1 summarizes the adjuvant studies that are being carried out in our institute. Details of the various study protocols have been described previously (2–4), and can be summarized as follows:

1. In a large majority of patients, the primary tumor was classified as T2a or T3a, N0 or N1 according to the UICC TNM International Classification. Very few patients classified as T1a were entered into these studies since there is a specific concomitant trial for this tumor stage (5);
2. All patients were first subjected to radical mastectomy (RM);

3. All patients had histologically positive axillary lymph nodes (N+);

4. No postoperative radiotherapy was administered;

5. Adjuvant chemotherapy was started within 4 weeks from surgery;

6. The entire treatment and followup studies were carried out at the Institute; and

7. Time and sites of primary treatment failure were assessed by critical reevaluation of roentgenograms. Whenever technically feasible, a tissue biopsy was obtained to confirm or rule out clinical findings.

TABLE 1.—Ongoing adjuvant studies for operable breast cancer with N+

Combined Treatment	Years of entry	Number of patients	Menopausal status
RM vs RM plus CMF (12 cycles).	6/73–9/75	386	Pre, Post
RM plus CMF: 12 vs 6 cycles.	9/75–5/78	460	Pre, Post
RM plus CMF-P (6 cycles) → AV (4 cycles) Dose intensification vs no intensification.	5/77-	109*	Post < 65 years
RM plus CMF (12 cycles) and tamoxifen.	5/77-	85*	Post > 65 years
RM plus CMF (12 cycles).	6/78	120*	Pre

*As of October 1979.

The first cyclophosphamide, methotrexate, 5-fluorouracil (CMF) trial was designed to study the impact on RFS and total survival rates of surgical adjuvant combination chemotherapy versus surgery alone. The second randomized study was started with the intent to determine whether the duration of adjuvant chemotherapy could be safely reduced without jeopardizing the results. In this trial, the accrual for postmenopausal patients was discontinued in May 1977, since the 3-year analysis of the first CMF study failed to confirm a significant impact of adjuvant CMF also in postmenopausal women (2). Based on the hypothesis that, in postmenopausal patients, adjuvant treatment should be more intensive, two trials were started in this age group, and patient accrual is still open at the time of writing. The design of the first of these trials included the sequential administration of two non-cross-resistant regimens such as the original NCI combination (cyclophosphamide, methotrexate, 5-fluorouracil, prednisone) (CMF-P) followed by the Adriamycin-vincristine (AV) regimen (6,7). Patients less than 65 years of age are randomly allocated to receive treatment at a consistent dosage or with progressive dose intensification. The dose intensification regimen (3) was partially based on the Norton-Simon mathematical model relating tumor size to sensitivity to therapy (8). In postmenopausal women older than 65 years, the ongoing trial in Milan includes 12 cycles on CMF combined with tamoxifen (20 mg daily for 1 year). While waiting for the final 5-year results of the first CMF study, premenopausal patients with N+ are presently treated with 12 cycles of CMF. The aim of this prospective study is to more systematically assess the prognostic importance of some clinical (bone scans) and biological (estrogen receptors, labeling index, histological features, hormone levels in blood and urines) parameters on treatment failure and total survival.

Statistical analyses were carried out utilizing the actuarial life table method to summarize both RFS and total survival distributions starting from the date of mastectomy. Findings are reported in the text and in the tables for one point in time (5 years for the first and 3 years for the second CMF study) as percentages of patients free of disease or surviving as derived from the life table plots. The P values quoted represent comparison of the entire curves and were assessed by the Mantel-Haenszel test for survivorship data (9).

THERAPEUTIC RESULTS

Therapeutic results are presently available only for the first two adjuvant studies (CMF versus control and CMF 12 versus 6 cycles).

Early 5-Year Results

The initial 5-year results of adjuvant CMF versus control are presented according to the parameters of stratification in table 2. The actuarial analysis confirms the earlier findings (2,3,10), ie, that 5 years after surgery, women treated by RM combined with adjuvant CMF have significantly higher RFS and total survival rates than women treated with surgery alone. Furthermore, the likelihood of remaining disease free at 5 years is higher in patients with minimal tumor cell burden in the axilla (one to three nodes) compared to patients with more extensive metastatic involvement (more than three nodes). On the contrary, the analysis of RFS related to menopausal status per se confirms that the therapeutic advantage of CMF chemotherapy remains significant in the pre- but not in the postmenopausal group. The 5-year results in premenopausal women are directly proportional to the degree of nodal involvement in the axilla. In fact, the RFS in patients with one to three nodes is 82.2% for the CMF group, compared to 51.3% for the control group ($P=0.0001$), while in patients with more than three nodes the findings are 39.7% for CMF and 28.8% for controls, respectively ($P<0.01$). The corresponding findings for total survival in premenopausal women are as follows: one to three nodes, 90.0% versus 68.3% ($P=0.008$); more than three nodes, 78.9% versus 46.5% ($P=0.05$).

TABLE 2.—Early 5-year results after adjuvant CMF (12 cycles) vs controls

	RM%	RM+CMF %	P
Relapse-free survival			
Total	48.2	63.5	0.0005
One to three nodes	53.3	70.5	0.0007
More than three nodes	35.6	45.3	0.0300
Premenopause	44.3	69.4	0.0001
Postmenopause	51.5	56.0	0.2200
Total survival	66.4	76.5	0.0400

In the majority of women with primary treatment failure, the first relapse was detected in distant sites. No appreciable differences were seen in the pattern of metastatic distribution between patients treated with surgery alone and those given adjuvant chemotherapy. Local-regional relapse as the sole apparent site of first recurrence was observed in 11.2% after mastectomy and in 7.7% after mastectomy plus CMF.

Influence of Amenorrhea and Dose Level

The hypothesis that adjuvant CMF is effectively "chemical castration" has been thoroughly discussed in previous publications (3,11,12). Table 3 summarizes the essential 5-year results showing once more that the RFS is similar in the groups with CMF-induced amenorrhea and those without. The fact that drug-induced amenorrhea is not tantamount to chemical castration is also supported by the results of therapeutic ovariectomy. In fact, ovariectomy induced objective regression in two of six women relapsing after adjuvant CMF, and in one of the two responders chemotherapy had induced amenorrhea. Response to ovariectomy followed 6 to 8 weeks later by AV chemotherapy was also observed in three of four patients relapsing after adjuvant CMF, and two of three responders had drug-induced amenorrhea at the time of castration (13). Finally, in our patients <35 years, ie, in an age group that is known to be almost refractory to therapeutic castration, the 5-year RFS was statistically superior in the 15 CMF-treated patients (60.1%) compared to 7 control patients (9.5%).

TABLE 3.—Percent 5-year RFS in premenopausal women related to drug-induced amenorrhea

	<40 years		>40 years	
CMF-amenorrhea	56.5	P=0.27	74.9	P=0.34
No amenorrhea	52.2		75.0	
Total CMF	50.9	P=0.02	76.1	P=0.0003
Total control	25.8		50.4	

The percent of protocol drug dosage administered has emerged as an important factor influencing RFS and total survival (10,13). More detailed analysis confirms that this observation is also true at 5 years. Table 4 shows that, both in advanced breast cancer and in the adjuvant setting, the therapeutic efficacy of CMF is dependent upon the amount of dose administered. To ensure an adequate evaluation of this clinical-pharmacological, ie, dose-response relationship, an analysis in clinically metastatic disease was carried out in a series of 57 consecutive women who in a previous randomized study were allocated to receive CMF chemotherapy (7). It is interesting to note that in women receiving less than 55% of the optimal dose none achieved complete or good partial response. Most important, both in advanced disease and in the adjuvant group, the results were almost superimposable between pre- and postmenopausal patients. However, in the adjuvant situation, the 5-year RFS was once more related to the extent of axillary involvement, and this was true within each dose group (table 5).

TABLE 4.—Response to CMF related to dose levels in advanced and early breast cancer

Percent of dose administered	Advanced disease CR+PR(%)	Adjuvant situation 5-year RFS (%)	
>85	66.7	79.3	P=0.04
84 to 55	48.4	59.1	
<55	0	50.0	
Low-dose CMF	*17.6	**47.5	
Total CMF	52.6	63.5	
Total control	-	48.2	

* > 60 to 65 years and/or extensive bone lesions.
** > 60 to 65 years.

TABLE 5.—Relationship between percent of adjuvant CMF administered and 5-year RFS in the two major nodal subgroups

Percent of dose administered	RFS (%) in nodal subgroups	
	1 to 3	3
>85	85.5	68.3
84 to 55	71.1	45.2
<55	58.3	33.3
Low-dose CMF*	57.6	22.2
Total CMF	70.5	45.3
Total control	53.3	35.6

*Postmenopausal patients > 60 to 65 years.

The observed difference in the total RFS between pre- and postmenopausal women could be related to the amount of drug administered rather than to menopausal status. Table 6 shows that twice as many postmenopausal women as premenopausal women received > 85% of the protocol dose (26.2% versus 13.5%), whereas almost the reverse occurred for the

dose level < 55% of the protocol dose (premenopausal 9.7% versus postmenopausal 25.0%).

TABLE 6.—Percent of pre- and postmenopausal women receiving various dose levels of adjuvant CMF

Percent of dose administered	Total (207)	Pre (103)	Post (104)
>85	19.8	26.2	13.5
84 to 55	62.8	64.1	61.5
<55	17.4	9.7	25.0
Low-dose CMF*			63.2 (31/49)

*Postmenopausal patients > 60 to 65 years.

The above-reported differences are mainly due to the fact that 31 of 49 postmenopausal patients (63.2%) who were older than 60 to 65 years received a low-dose CMF (methotrexate 30 mg/m², fluorouracil 400 mg/m²). The low-dose regimen was not specifically required by the original protocol design but it was applied as a caution against potential severe and/or prolonged myelosuppression. Experience revealed that CMF was not a severe myelosuppressive combination and bone marrow toxicity was not more frequent in post- than in premenopausal women. In fact, while leukopenia and/or thrombocytopenia were detected in almost all patients, repeated episodes of transient myelosuppression requiring a temporary dose reduction in more than 9 of 12 CMF courses occurred in only 4% of postmenopausal and 3% of premenopausal women.

CMF 12 Versus 6 Cycles

The preliminary 3-year results of the trial comparing in premenopausal women 12 versus 6 cycles of adjuvant CMF are summarized in table 7. In short, both RFS and total survival rates were not significantly affected by the duration of treatment. Also in this trial, RFS was not statistically influenced by CMF-induced amenorrhea, and this finding further supports our previous conclusion. Furthermore, the fact that estrogen receptor (ER) assay was carried out in more than half of patients allowed the correlation of ER status with 3-year RFS. As can be seen from table 7, ER+ patients showed a survival rate that was similar to that of ER− patients (P=0.23). In particular, the RFS for both ER+ and ER− tumors was comparable between patients with and without CMF-induced amenorrhea (4). Analysis of the results dealing with CMF 12 versus 6 cycles in postmenopausal women and the detailed analysis of the dose-response relationship are still in progress.

TABLE 7.—Summary of the 3-year results of CMF 12 vs 6 cycles in premenopausal women

	CMF 12	CMF 6	P
Relapse-free survival			
Total	85.4	82.6	0.29
1 to 3 nodes	92.3	88.3	0.22
>3 nodes	69.7	71.0	0.36
Amenorrhea	84.6	86.1	0.43
<40 years	85.4	71.2	0.12
>40 years	84.1	87.2	0.25
No amenorrhea	73.6	68.8	0.32
<40 years	73.6	65.4	0.11
>40 years	100.0	100.0	
ER+	82.9		P=0.23
ER−	78.2		
Total survival	86.2	85.1	0.49

Acute and Delayed Toxicity

The incidence of acute toxic manifestations has been reported previously (2-4,14). Mild nausea and vomiting occurred in most patients with each iv drug exposure. Altogether, loss of hair was moderate, and frank alopecia occurred in less than 10% of women. In neither above-mentioned series was bone marrow toxicity cumulative. Severe leukopenia and/or thrombocytopenia were rare (10% to 15%) and no patient required leukocyte or platelet transfusion. There was no incidence of treatment-induced infection. Transient episodes of oral mucositis, conjunctivitis, and chemical cystitis were few, mild, and promptly reversible upon temporary drug discontinuation. A transient mild to moderate elevation of serum alkaline phosphatase, SGOT, SGPT, and LDH was observed in about 1% of patients, either during CMF administration or soon thereafter. These biochemical abnormalities were never sufficiently severe to necessitate discontinuation of therapy.

Although it is premature to evaluate delayed toxicity, no patient has so far shown any evidence of biochemical, radioisotopic, or histologic evidence of chronic liver toxicity attributable to prolonged MTX administration. We also have no evidence that CMF has increased the incidence of second neoplasms. In particular, no acute leukemia has been observed. A short description of the histological type of second tumors detected so far in the first CMF series was reported by Bonadonna et al (13). In the control group the incidence of second breast tumors was 2.2%, and that of other second tumors was 1.7%. The corresponding findings for the CMF group were 1.0% and 1.9% respectively.

Discussion

Consecutive analyses of our adjuvant studies are gradually confirming that CMF combination is an effective adjuvant treatment and that a significant improvement of RFS as well as of total survival can be prolonged at least up to the fifth year from mastectomy. Though this is still an early finding, this observation represents an important scientific and professional achievement, for no other therapeutic initiative has significantly altered the 5-year results of operable breast cancer with positive axillary nodes in the past few decades. Thus our data confirm and expand the pioneering studies of Martin, Skipper, Schabel, Fisher, and Nissen-Meyer (1).

We leave more detailed description of treatment subsets, pattern of disease recurrence, and value of secondary treatments to our next report. However, the importance of adequate dose level deserves a brief discussion. Our results indicate that in the treatment of advanced breast cancer, as well as in the adjuvant situation, there is a dose-response relationship, at least with CMF chemotherapy. These findings, which are in agreement with those previously described in experimental animal systems (15), repudiate the concept that the therapeutic effect of adjuvant combination chemotherapy is different between pre- and postmenopausal women. Furthermore, the dose-response relationship showing that about 80% of optimally treated patients remain disease-free at 5 years regardless of their menopausal status disproves the hypothesis that the favorable results of adjuvant chemotherapy are predominantly due to "chemical castration" (12). The importance of the percent of dose administered will be better defined by our adjuvant study with sequential combination chemotherapy (CMF-P-AV). In fact, in this trial no dose reduction is utilized in the presence of myelosuppression, but subsequent treatment is delayed until full bone marrow recovery occurs. Moreover, one treatment arm receives a progressive dose intensification schedule (3).

Other important observations are emerging from our second CMF adjuvant trial. Provided the amount of drug administered is high, there is probably no real advantage in prolonging CMF chemotherapy beyond the sixth month.

Should the 3-year results be confirmed in subsequent analyses, prolonged adjuvant treatment could be theoretically justified only if non-cross-resistant regimens are utilized. Finally, ER status does not predict the therapeutic effect of adjuvant CMF (16). This observation would further suggest that the actual influence of the numerous biological parameters remains to be better defined. For the time being, present results are largely, if not exclusively, interpretable as a direct effect of chemotherapy given at maximum tolerated dosage. Pretreatment compromises, such as low-dose CMF or leukeran, methotrexate, 5-fluorouracil, and prednisone (LMF [P]) schedule (17), have already been proven to be followed by negative results.

REFERENCES

1. Bonadonna G, and Valagussa P. Adjuvant therapy of primary breast cancer. *In* Principles of Cancer Treatment (Carter SK, Glatstein E, and Livingston RB, eds). New York, NY, McGraw-Hill, 1980. In press.
2. Bonadonna G, Valagussa P, Rossi A, et al. The CMF program for operable breast cancer with positive axillary nodes. Updated analysis on the disease-free interval, site of relapse and drug tolerance. Cancer 39: 2904–2915, 1977.
3. Bonadonna G, Valagussa P, Rossi A, et al. Are surgical adjuvant trials altering the course of breast cancer? Semin Oncol 5:450–464, 1978.
4. Tancini G, Bajetta E, Marchini S, et al. Preliminary 3-year results of 12 vs 6 cycles of surgical adjuvant CMF in premenopausal breast cancer. Cancer Clin Trials 2:285–292, 1979.
5. Veronesi U.Value of limited surgery for breast cancer. Semin Oncol 5:395–402, 1978.
6. Canellos GP, DeVita VT, Lennard Gold G, et al. Combination chemotherapy for advanced breast cancer: response and effect on survival. Ann Intern Med 84:389–392, 1976.
7. Brambilla C, De Lena M, Rossi A, et al. Response and survival in advanced breast cancer after two non-cross-resistant combinations. Br Med J 1:801–804, 1976.
8. Norton L, and Simon R. Tumor size, sensitivity to therapy, and design of treatment schedules. Cancer Treat Rep 61:1307–1317, 1977.
9. Mantel N. Evaluation of survival data and two new rank oruer statistics arising in its consideration. Cancer Chemother Rep 50:163–170, 1966.
10. Rossi A, Bonadonna G, Tancini G, et al. Trials of adjuvant chemotherapy in breast cancer. The experience of the Istituto Nazionale Tumori of Milan. Eur J Cancer. In press.
11. Bonadonna G. Adjuvant castration versus adjuvant chemotherapy. Int J Radiat Oncol Biol Phys 4:918–919, 1978.
12. Bonadonna G, Valagussa P, De Palo G. The results of adjuvant chemotherapy in breast cancer are predominantly due to the hormonal change such therapy induces. The view against. *In* Controversies in Medical Oncology (Scoy-Mosher V, ed). Boston, MA, GK Hall & Co. In press.
13. Bonadonna G, Valagussa P, Rossi A, et al. CMF adjuvant chemotherapy in operable breast cancer. *In* Adjuvant Therapy of Cancer II (Salmon SE, and Jones SE, eds). New York, NY, Grune & Stratton, 1979, pp 227–235.
14. Bonadonna G, Brusamolino E, Valagussa P, et al. Combination chemotherapy as an adjuvant treatment in operable breast cancer. N Engl J Med 294:405–410, 1976.
15. Skipper HE, and Schabel FM (jr.). Quantitative and cytokinetic studies in experimental tumor systems. *In* Cancer Medicine, 2d ed (Holland JF, and Frei E (III), eds). Philadelphia, PA, Lea & Febiger. In press.
16. Bonadonna G, Valagussa P, Tancini G, et al. Estrogen receptor status and response to chemotherapy in early and advanced breast cancer. Cancer Chemother Pharmacol 4:37–41, 1980.
17. Senn HJ, and Mayr AC. Adjuvant chemotherapy in breast cancer–Swiss co-operative studies. Cancer Treat Rev (Suppl) 6:79–85, 1979.

Prolonged Disease-Free Survival in Advanced Breast Cancer Treated with "Super-CMF"Adriamycin: An Alternating Regimen Employing High-Dose Methotrexate with Citrovorum Factor Rescue [1]

I. Craig Henderson,[2]* Rebecca Gelman,[3] George P. Canellos,[2] and Emil Frei, III[2,4]

SUMMARY

Sixty-six patients were treated with the combination "super-CMF"as follows: cyclophosphamide 100 mg/m² po days 1 through 14; methotrexate 3.0 g/m² iv days 1 and 8; 5-fluorouracil 600 mg/m² iv days 1 and 8; citrovorum factor rescue 10 mg/m² iv days 2 and 9 and po days 2 through 4 and 9 through 11; and Adriamycin 70 to 90 mg/m² iv day 29. This cycle was repeated at 49-day intervals. The single-agent activity of high-dose methotrexate with rescue was first evaluated in breast cancer patients in a truncated phase II study; 3 of 19 had a partial response (> 50% regression of measurable lesions) after 2 to 6 weekly courses and another 6 to 19 had some objective evidence of response (> 25%, < 50%). The response rate to the combination "super-CMF" was: complete response (CR) 15 of 55 evaluable patients (27%) and partial response (PR) 28 of 55 (51%). The median duration of response was 24.5 months and the median time to treatment failure was 21.6 months. Nineteen of these patients have completed 12 to 18 months of drug treatment and have been followed in continuing remission for 1 to 28 months without further therapy. The total response rate and the CR rate achieved with this regimen are substantial, and the durability of the responses appears to be considerably greater than that obtained with other drug combinations.

[Cancer Treat Rep 65 (Suppl. 1): 67–75, 1981]

Although breast cancer responds to many single agents, four appear more active than the rest: cyclophosphamide (C), methotrexate (M), 5-fluorouracil (F), and Adriamycin (A or Adria) (1). Multiple combinations of any three of these four in various dose schedules predictably induce remissions in 50% to 80% of treated patients (1–6). Since it has previously been shown in the treatment of leukemia and lymphomas that significant prolongation of survival or "cure" is possible only after the development of regimens capable of inducing complete remissions in a large percentage of treated patients (7), it is reasonable to assume that such an increase in the CR rate must be the next step in the successful treatment of patients with metastatic breast cancer. Further, this goal does not appear obtainable using conventional doses and schedules of available drugs.

In animal tumor models there is a steep dose-response to each of these four drugs (8,9). Although this relationship between dose and response has not been clearly established in human solid tumors, in tumor systems such as the AKR mouse leukemia model small increments in drug dose may result in a several log increase in the tumor cell kill from each course of therapy.

This pilot study was designed to explore the potential for significant dose escalation in the treatment of human breast cancer by modification of the well-established "CMF" regimen (3,10,11). In the regimen described here the dose of M has been increased 50-fold, and citrovorum rescue is used to prevent toxicity. As a result, the usual doses of C and F may be administered with high-dose methotrexate (M). Since the concomitant use of Adria with C and F would

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require significant dose reductions of all these drugs, Adria has been given on day 29 of a 49-day cycle. In this way the Adria can be administered at its maximally tolerated dose after bone marrow recovery from initial therapy with CMF (fig 1).

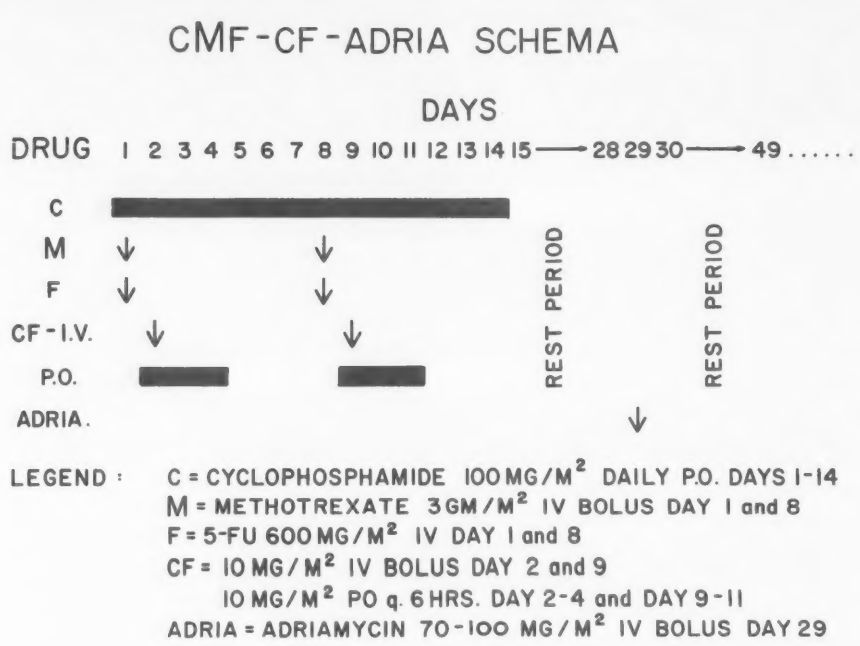
CMF-CF-ADRIA SCHEMA
DAYS
DRUG 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 28 29 30 49 .....
C
M
F
CF-I.V.
P.O.
ADRIA.
REST PERIOD
REST PERIOD
LEGEND : C = CYCLOPHOSPHAMIDE 100 MG/M² DAILY P.O. DAYS 1-14
M = METHOTREXATE 3 GM/M² IV BOLUS DAY 1 and 8
F = 5-FU 600 MG/M² IV DAY 1 and 8
CF = 10 MG/M² IV BOLUS DAY 2 and 9
10 MG/M² PO q. 6 HRS. DAY 2-4 and DAY 9-11
ADRIA = ADRIAMYCIN 70-100 MG/M² IV BOLUS DAY 29

FIGURE 1.—Schema for combination CMF-CF-Adria.

PATIENTS AND METHODS

High-dose M with citrovorum rescue has not previously been demonstrated to be active in the treatment of breast cancer. To confirm that rescued M is active in this disease, 31 patients with breast cancer were given 1 to 3 g/m² of M at weekly intervals. Citrovorum factor was given by iv bolus exactly 24 hours after the M dose and continued orally every 6 hours for 3 days. At the first evidence of *any* objective response or after at least three and no more than six doses, the patients who were otherwise eligible for treatment with the regimen "super-CMF" (12 of 31 patients) were begun on combination chemotherapy. This precluded determination of CR and PR rates as might have been done in a more conventional phase II study, since single-agent M was not continued until a maximum response had been achieved.

Sixty-six patients with biopsy-proven breast cancer were entered into a study of the full combination between December 10, 1975, and September 1, 1979. All patients had measurable disease in skin, breast, lymph nodes, pulmonary parenchyma, or liver. During the first year of the study patients with only bone lesions were excluded, but subsequently such patients were included if all lesions were lytic and easily followed with serial X-rays. Patients whose only sites of disease consisted of a pleural effusion or CNS metastasis were excluded. Patients were not excluded because of prior therapy unless they had received a cumulative dose of C exceeding 1.5 g/m² or Adria exceeding 100 mg/m². Patients with evidence of compromised renal function, usually defined as a creatinine clearance < 60 cm³/minute, were excluded, and patients considered to have a life expectancy of less than 3 months were not treated. In the original protocol design it was anticipated that patients beyond age 65 would not tolerate therapy; in actuality eight patients over 65 were entered into the study, but the two oldest, ages 73 and 76, were promptly taken off therapy after one course because of minor toxicities and concern on the part of the primary care physicians that the patients could not manage such a complicated regimen. All patients gave appropriate informed consent.

In addition to the 66 patients treated under the protocol, another 18 patients who were not eligible for the study received CMF with or without Adria. Most of these patients were ineligible because of prior C or Adria treatment; several had relapsed after adjuvant therapy. Four patients in this group had no measurable disease, three had marginal renal function, and in one the tumor type was unknown. These patients were included in the analysis of drug toxicities even though they were not evaluable for response.

CR was defined as disappearance of all clinical, laboratory, and radiologic evidence of active disease for a period of at least 1 month. Skin lesions, lymph nodes, or breast masses with remaining induration or any other suggestion of residual disease were biopsied to confirm a clinical impression of regression. Bone lesions were scored as a CR only with a normal bone scan or evidence that a lytic lesion had returned to a normal appearance. PR was defined as a greater than 50% reduction in the product of two perpendicular dimensions or a 50% reduction in the squared diameter of a circular lesion; all PR's lasted for at least 1 month without evidence of concurrent progression at any other metastatic site. Bone lesions were scored as a PR only when lytic-to-blastic changes could be documented. Although 29 patients had elevations of CEA in excess of 5.0, change in CEA was never the basis of scoring a patient as a complete or partial responder. On the other hand, the CEA returned to less than 5.0 in all complete responders and fell toward normal in all partial responders. A patient was considered to have improvement if there was objective evidence of tumor regression that had not reached 50%, but in one patient the only objective evidence of improvement was a fall in CEA and stabilization of other disease sites. Patients were considered to have had stable disease if there was no evidence of progression on two measurements a month or more apart. Progressive disease was defined as the appearance of any new lesion or any objective increase in the size of an existing lesion any time after the first month of therapy in a patient who had not previously had a documented response or documented disease stabilization.

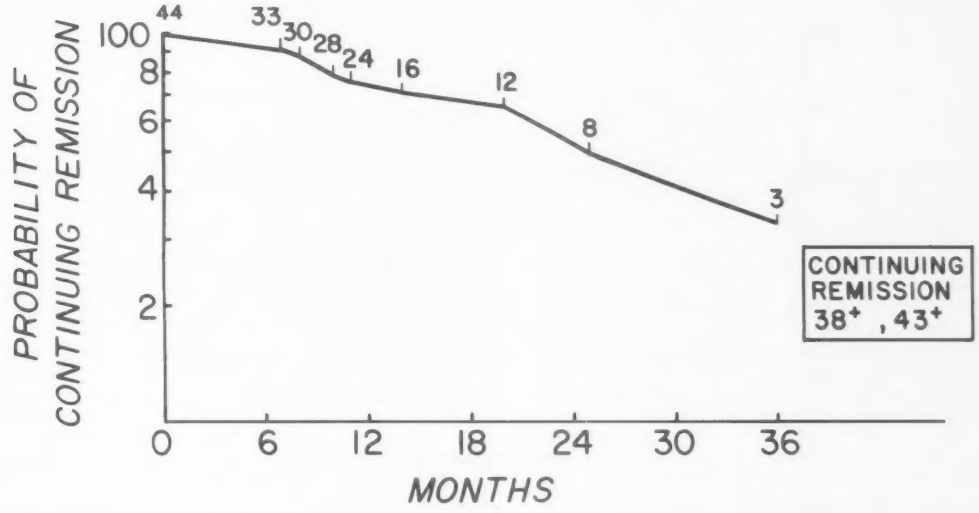

FIGURE 2.—Duration of response calculated as interval from first objective evidence of response to disease progression for all patients with a CR or PR.

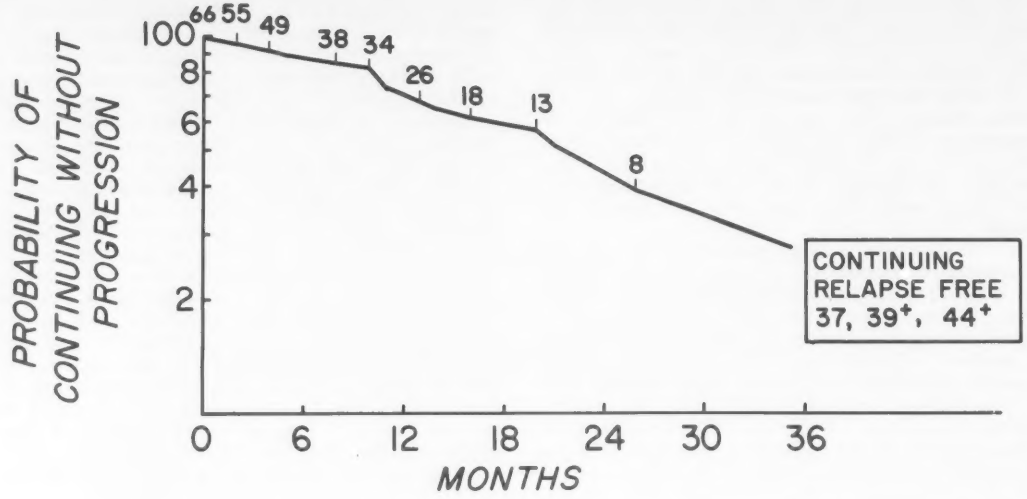

FIGURE 3.—TTF calculated as interval from initiation of therapy to disease progression for all patients regardless of response or evaluability.

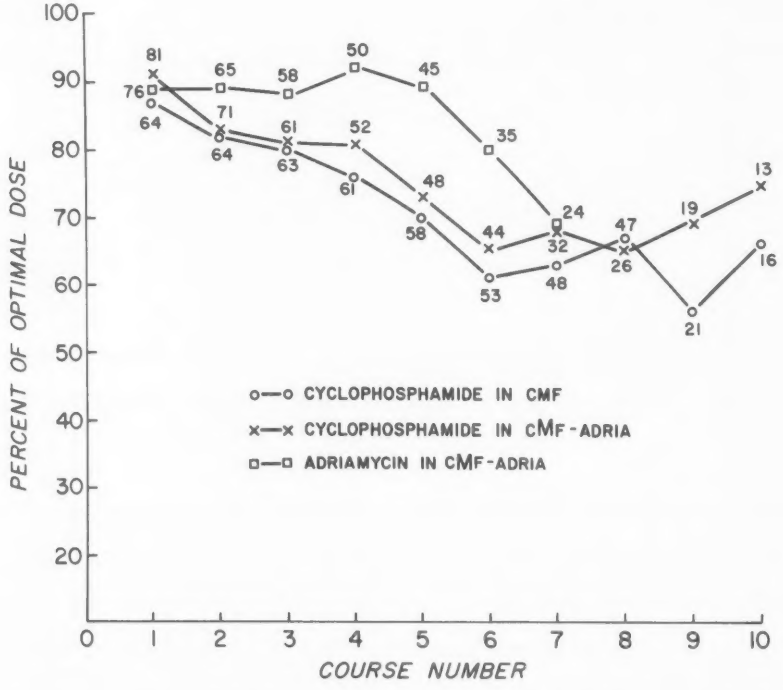

FIGURE 4.—Comparison of the average percent of optimal dose administered for patients treated either on this study with "super-CMF" or on an adjuvant protocol utilizing conventional CMF: C 100 mg/m² po days 1 to 14, M 60 mg/m² iv days 1 and 8, F 600 mg/m² iv days 1 and 8. The average percent of optimal dose was calculated for C given with "super-CMF," C given with adjuvant CMF, and Adria given with "super-CMF." Numbers indicate patients followed to point of vertical slashes.

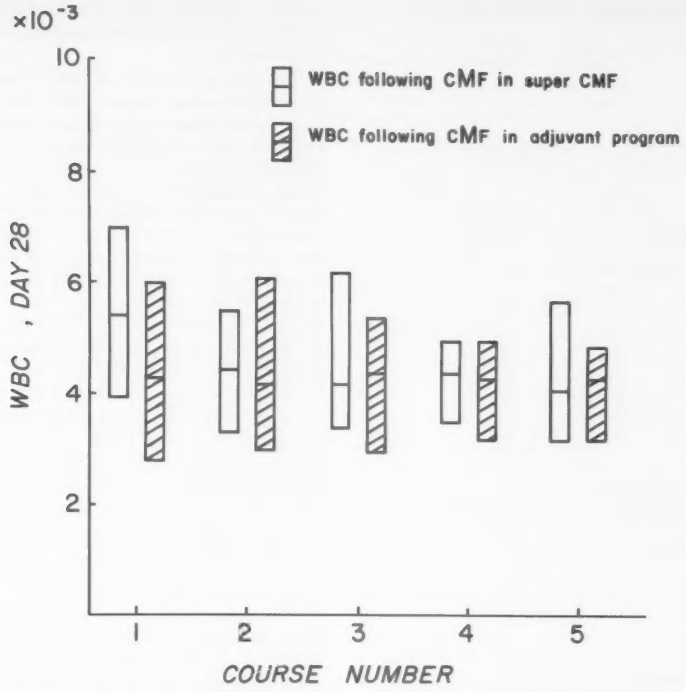

FIGURE 5.—Comparison of day 28 wbc count following treatment either with CMF in the "super-CMF" regimen ☐☐ or with conventional CMF ▨▨ as described in figure 4. Bars indicate median and range of wbc counts between 25% and 75% of total array.

The duration of response (fig 2) and the time to treatment failure (fig 3) curves and medians were determined by the life table method of Kaplan and Meier (12,13). The doses of C administered in the "super-CMF" and conventional CMF regimens were compared, and the differences in average dose were evaluated using the normal approximation of the binomial test (fig 4); differences in the myelosuppression resulting from each of these treatments were assessed with a generalized Wilcoxon test (fig 5) (14). The response rates of patients with various characteristics (table 1) were compared using Fisher's exact test (15).

The "super-CMF" regimen was administered in a 49-day cycle (fig 1). The M dose was initially set at 1 g/m², but the protocol was later amended and the dose of M increased to 3 g/m² to maximize CNS levels. All but the first 171 courses of M were at 3 g/m². Sodium bicarbonate, 3.2 g orally every 3 hours, was begun 12 hours before and continued for 48 hours after each M dose to alkalinize the patient's urine. If the urinary pH fell below 7.0, supplemental sodium bicarbonate was given iv just before M administration; otherwise 1 liter of normal saline was given just before high-dose M to ensure adequate hydration and to further diminish the possibility of renal toxicity. F was given by iv bolus 30 to 60 minutes before M. All patients had serum creatinines drawn at 0 and 24 hours and M levels at 24 hours to monitor for possible M toxicity. Citrovorum factor was given 24 hours after each dose as described above when M was used alone. The other drugs utilized in this regimen were given in a conventional fashion at the doses and schedules outlined in figure 1. During the first course each patient was given 70 mg/m² of Adria, but this dose was escalated in subsequent courses until the day 42 (day 14 post-Adria) wbc count nadir was between 1,000 and 2,000. It was initially anticipated that patients might safely receive a single dose of Adria as high as 100 mg/m², but in fact no patient was given more than 95 mg/m². Adria was discontinued when a cumulative dose of 490 mg/m² had been given, and at that point, patients continued to receive CMF on a 28-day cycle until each had received a total of 15 months of therapy.

Six patients with ascites, pleural effusions, or extensive liver involvement were begun on day 29 of the first cycle with a single dose of Adria rather than with CMF, since it seemed plausible that the initial Adria might result in early and rapid disease regres-

TABLE 1.—Characteristics of treated patients

Median age	52 years	Range	24–76
Median DFI	17 months	Range	0–168

	At diagnosis	At time of therapy
Menstrual status		
Premenopausal	48%	23%
Perimenopausal (<1 year)	5%	13%
Postmenopausal (>1 year)	47%	64%

	Number of patients
Estrogen receptor protein	
"Negative" or <10 fmol/mg protein	17
"Positives" or >10 fmol/mg protein	19
Unknown	30
Prior therapy	
Oophorectomy	6
Ardrenalectomy/hypophysectomy	12
Tamoxifen, estrogens, androgens	16
Chemotherapy	14
No prior systemic therapy	35
Sites of metastases	
Skin and soft tissue	6
Visceral disease	42
Only one involved site	25
>three involved sites	18

Median duration of metastases prior to therapy: 2 months Range 0–71

sion, a slightly improved performance status, or a decrease in third-space fluid, and consequently decreased toxicity from high-dose M.

RESULTS

Of the 31 patients given high-dose M as a single agent, 7 received less than three weekly doses, 11 received three to five doses, 7 were given six doses, and 6 received more than six courses. Nineteen of these patients were evaluable for response: three had a PR (>50% regression of measurable lesions) and another six had objective evidence of disease regression (>25%, <50%), which did not fulfill criteria for a PR. There were no discernible differences in the response rate or the quality of response in patients treated with either 1 g/m² or 3 g/m² of M, but the number of evaluable patients and the short duration of single agent therapy precluded a meaningful conclusion about dose-response to M. Half of the inevaluable patients were given less than three cycles and were removed from study prematurely through a misunderstanding of the protocol by primary care physicians. Other patients were inevaluable because of concomitant DES administration (one patient), uncertain tumor type (one), and early withdrawal associated with toxicity and failure to evaluate disease sites at the time the patient was removed from study (four patients).

The characteristics of the 66 patients treated with the combination "super-CMF" are outlined in table 1. In general these patients had had limited prior therapy, and all were ambulatory with a life expectancy estimated to be in excess of 3 months. Twenty-nine of these patients were treated with this regimen immediately after the demonstration of metastases, and 16 had initially presented with metastases (ie, DFI = 0). Even though these patients had an excellent performance status, almost two-thirds had visceral disease with involvement of liver, lung, or CNS; more than one-fourth had widely disseminated disease with more than three involved sites; about half of the patients with an estrogen receptor protein (ERP) determination were ERP negative.

Fifty-five patients are evaluable for response to the combination "super-CMF" (table 2). Fifteen had a CR (27%), and nine are in continuing complete remission at the present time. Another 28 patients (51%) had a PR. The total response rate is 78%. Only four patients (7%) had progression of disease in spite of therapy. Responses were seen in all evaluable sites (table 3), and included 88% of the bone lesions, 63% of lung parenchymal lesions, and 77% of liver metastases. There were no significant differences in the response rates of postmenopausal compared to premenopausal patients, of ERP positive compared with ERP negative patients, and of patients with a disease-free interval of less that 2 years compared with patients with a disease-free interval of more than 2 years.

TABLE 2.—Response to therapy

Patients evaluable for response	55
CR	15 (27%)
PR	28 (51%)
No response	12 (22%)
Improved	6
Stable disease	2
Progressive disease	4
Patients inevaluable for response	11

Eleven patients were not considered evaluable for response. Five patients had mild toxicity, such as an asymptomatic fall in creatinine clearance or mild nausea and vomiting, and withdrew 1 to 6 weeks after initiation of the first drug dose; all were stable at the time they were withdrawn from the study. Four patients died after 1 to 4 weeks on the study; two of these had massive pulmonary emboli, one died of hepatic failure, and another died of respiratory failure not clearly related to either disease progression or drug toxicity. Finally, one patient was lost to followup since she left the area 3 weeks into the study, and one patient received "palliative radiotherapy" to all sites of measurable disease 1 week after initiation of "super-CMF."

TABLE 3.—Response by disease site

Site	N*	Eval.**	CR	Responses†	
				PR	NR
Bone	35	26	9 (35%)	14 (54%)	3 (12%)
Lung	24	19	7 (37%)	5 (26%)	7 (37%)
Liver	15	13	5 (38%)	5 (38%)	3 (23%)
Skin	12	11	4 (36%)	2 (18%)	5 (45%)
Lymph nodes	15	15	10 (67%)	3 (20%)	2 (13%)
Breast	11	11	4 (36%)	6 (55%)	1 (9%)
CNS	2	0	–	–	–

*Number of patients with any involvement at this site.
**Number of patients evaluable at this site, even if inevaluable at other sites.
†Number responding. ( ) = number responding/number evaluable.

Two patients had CNS metastases prior to protocol entry; both patients received whole brain irradiation prior to initiation of any drug treatment and were in remission from neurological symptoms at the time of entry into the study. One of these patients subsequently had progression of her CNS disease along with progression of disease at other sites, and the other patient is in continuing remission at 44 months. Two patients free of any neurological symptoms when entered on study had a CNS relapse without evidence of progression of disease at any other sites.

In general, drug therapy has been discontinued regardless of the patient's response category after 15 months, but this ranged from 12 to 18 months. One patient discontinued her treatment at 8 months while in complete remission. A total of 19 patients has been observed for periods from 1 to 28 months after completion of all drug therapy. Eleven of these patients were in complete remission at the time of drug discontinuation, and six of these eleven patients are in continuing complete remission with periods of follow-up from 1 to 28 months. Seven of the eight patients whose therapy was stopped while in partial remission are in continuing partial remission 2 to 21 months later.

The disease characteristics and experience of the complete responders are outlined in table 4. The probability of a complete remission and the durability of the response does not appear to be related to the initial sites of disease or the number of involved sites; 10 of 42 patients with visceral disease and 7 of 18 patients with more than three involved sites had a complete response. In all cases relapses were in the sites of prior disease.

The response duration determined by Kaplan-Meier plot is shown in figure 2. The median duration of response (CR+PR) is 24.5 months (95% confidence limits 10.0, 36.0). Of the 44 responders, 13 have relapsed. The median duration of response for complete responders is 26.2 months. The median duration for partial responders cannot yet be estimated, and it is still not possible to determine if the response duration of the complete responders will be significantly different from that of the partial responders. The

TABLE 4.—Status of complete responders

Sites of disease at initiation of therapy	Months on study*	Sites of relapse
CNS, bone, lung	44+	–
Bone, liver, nodes, breast, CEA	37	Bone, nodes, breast, CEA
Liver, CEA	33+	–
Lymph nodes	30+	–
Bone, lung, nodes, CEA	26	Bone, CEA
Bone, lung, liver, CEA	26	Lung, CEA
Bone, lung, nodes	22+	–
Skin	21	Skin
Bone, skin, nodes	15+	–
Bone, lung	15+	–
Bone, breast	14+	–
Lung	**13+	–
Bone, lung, nodes, breast	13	Lung, breast, skin
Liver, CEA	8+	–
Skin	4+	–

*Time from initiation of therapy to relapse or to date of analysis if patient in continuing remission.
**Patient died of other causes; no breast cancer found at autopsy.

time to treatment failure (TTF) is plotted in figure 3; the median TTF is 21.6 months (90% confidence limits 8.0, 37.0). Twenty-one of the sixty-six patients on the protocol have had a treatment failure. Seventeen patients died, including two patients who had a CR and six patients with a PR.

TOXICITY

Over two-thirds of the patients treated with this regimen have received all therapy as outpatients. Only previously adrenalectomized patients and older patients have been routinely hospitalized for high-dose M treatment. The most important immediate toxicity has been nausea and mild vomiting associated with a large sodium bicarbonate dosage. Mucositis has been observed only rarely and was mild. Alopecia is total and occurs in 100% of the patients.

Myelotoxicity did not appear to differ from that seen in patients treated in this institution with adjuvant CMF during the same time period. In figure 4 the average dose of C and the average dose of Adria administered with each course of "super-CMF" has been plotted beside the average C dose administered with each course of adjuvant CMF. No statistically significant differences in the frequency or size of dose reduction are seen. In addition, when the median day 28 wbc counts and the 25% to 75% for the wbc count are plotted by course for the patients treated with either "super-CMF" or adjuvant CMF (fig 5), no significant differences in any but the first course are apparent using the two-sample Wilcoxon test. In the first course, the myelosuppression from adjuvant CMF was greater ($P=0.021$).

A wbc count nadir of less than 1,000 was seen with only 8 (2%) of the courses of CMF administered at full dose and in 11 (6.5%) of the full Adria courses

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(table 5). One episode of life-threatening, nonfatal sepsis was observed, and there were two fatal episodes associated with rapidly progressive disease. No significant bleeding has occurred, and no clinically important renal failure was seen. Less than 1% of the M courses have been associated with a 24-hour creatinine elevation exceeding 50% (table 5). No hemorrhagic cystitis has been observed.

TABLE 5.—Toxicity

	CMF	Adriamycin
Evaluable courses	354	208
Courses at 100% dose	274(77.0%)	167(80.0%)
WBC nadir <500	0	1(00.5%)
500–1000	8(02.0%)	10(06.0%)
1001–2000	64(33.0%)	65(39.0%)
2001–4000	123(45.0%)	77(46.0%)
>4001	79(29.0%)	14(08.0%)
Platelet nadir <20,000	0	2(01.2%)
20–50,000	5(02.0%)	4(03.0%)
50–100,000	16(06.0%)	12(08.0%)

	High-dose M in combination
Evaluable courses	625
Symptomatic renal failure	0
24-hour creatinine elevation	
20–50%	115(18.0%)
50–99%	4(00.6%)
> 100%	0

Three patients have developed a nonfatal cardiomyopathy and have been successfully treated with digoxin and diuretics. All three are asymptomatic after 1 to 24 months' followup. A pulmonary infiltrate in one patient was considered on histologic examination to be compatible with M pulmonary toxicity. Two episodes of acute hepatic decompensation have been observed. In one of these patients a clinical picture similar to acute yellow atrophy but associated with myelosuppression and mucositis appeared 1 week after initiation of the first course of CMF. The second episode of acute hepatic decompensation was fatal and occurred 1 week after a single, initial dose of Adria in a patient with massive hepatomegaly. Finally, unexplained seizures were observed in two patients shortly after receiving high-dose M. Neither of these patients has evidence of brain metastases, nor have brain metastases appeared subsequently. Both patients received further courses of high-dose M without difficulty.

DISCUSSION

This phase II study has demonstrated that single-agent high-dose M with citrovorum rescue is active in the treatment of breast cancer. Although the data do not permit a conclusion that high-dose M is either superior or inferior to more conventional doses, there was an objective response (> 25%) in 9 of 19 patients. This is in a range comparable to that achieved with lower doses of M (1).

The response rate obtained with the combination "super-CMF" is as high as that obtained with CMF, C/A, or CAF. The CR rate of 27% is actually somewhat higher than that reported in most previous studies (2,6), but the differences are not great enough to expect that it would be significant in a randomized trial comparing "super-CMF" with CMF or CAF.

A more significant finding is the long duration of the responses achieved with this regimen. Nineteen patients off all therapy have been followed for periods up to 28 months, including six patients who have been off therapy for more than 1 year. The median duration of response is 25 months, or about 10 to 12 months longer than that previously reported for most other regimens utilizing these drugs (2–6). Similarly, the median TTF, 22 months, is 12 to 15 months longer than that observed in an earlier study (5). The TTF is a particularly useful interval because all patients are included regardless of definitions of response or evaluability.

An original objective of this study was both a high CR rate (> 40%) and a median duration of remission in excess of 12 months. It was assumed that the completeness of the response and the durability of response would be related as in previous experience with hematologic malignancies (8). In fact, there appears to be some disparity in these two parameters of response in this study. This may be explained by the observation that all eight patients followed off therapy who had a PR at the time drug was discontinued had an "incomplete" response in bone or visceral areas where biopsy confirmation of a CR is difficult. It is very likely that the response scores assigned to these patients have underestimated the total tumor cell kill achieved with this regimen. The wide distribution of breast cancer metastases, the frequent involvement of bone, and the scirrhous or fibrotic nature of the lesions all make accurate evaluation of the response to therapy difficult. As a result, comparisons between different regimens based entirely on response rates are apt to be misleading.

The toxicity associated with this regimen is significant but not clearly greater than that observed when full doses of CMF or CAF are used. A major impediment, which is difficult to quantify but which may be dose limiting in some cases, is patient and physician inconvenience. The frequent administration of sodium bicarbonate (the major inducer of emesis in this regimen) and citrovorum rescue tablets requires more than average patient cooperation. On the other hand, patients were able to receive the majority of their care and followup in their own communities because of the long cycle length; many patients were seen at the Sidney Farber Cancer Institute on days 1 and 2 and days 8 and 9 only, and returned to their own communities during the remaining 40 days in the cycle. The number of patients excluded from this protocol because of inadequate renal function was small, but as a consequence almost no renal toxicity

was observed among these patients. Any life-threatening or fatal toxicities associated with this regimen did not appear to be uniquely related to the use of high-dose M or high-dose Adria.

Excellent responses were seen in all sites, including osseous metastases. Bone responses usually require recalcification of lytic lesions and can rarely be appreciated in the first 6 months after initiation of therapy. Since one-third of patients treated with "super-CMF" completed more than a year of therapy and another one-fourth are still on therapy and on study, this response rate in bony sites is not surprising.

Of these 55 patients, 2 developed new CNS metastases while on this regimen, an incidence which is probably not significantly different from the 5 of 105 CNS recurrences observed in another study utilizing drugs that do not cross the blood brain barrier (3). It is known that M administration in these doses may result in tumoricidal CSF levels, and systemic M with citrovorum rescue has been used successfully to treat CNS lymphoma and leukemia (16). It may be that successful eradication of the micrometastases of breast cancer might be possible with either still higher doses of M or more frequent administration of drug, since in the "super-CMF" regimen significant drug levels would have been reached in the CNS no oftener than twice every 49 days.

CONCLUSION

This study does not necessarily establish the superiority of the more intense regimen over conventional dose chemotherapy. Further, it does not establish that high-dose M is superior to more conventional doses, especially since the dose of Adria has also been escalated in this cyclic combination. On the other hand, in the context of a recent report demonstrating significantly better disease-free survival for patients given full doses of adjuvant CMF in comparison to patients who received less than 85% of scheduled doses (17), and a retrospective study that found higher response rates in patients with metastatic breast cancer who received more than 85% of an optimal CMF dose in contrast to those receiving either 65% to 85%, or less than 65% of scheduled doses (18), the very durable responses observed with "super-CMF" suggest there is a dose-response effect in human breast cancer. This question needs to be carefully addressed in large phase III trials.

REFERENCES

1. Henderson IC, and Canellos GP. Cancer of the breast: the past decade. N Engl J Med 302:17–30 and 78–90, 1980.
2. Brunner KW, Sonntag RW, Martx G, et al. A controlled study in the use of combined drug therapy for metastatic breast cancer. Cancer 36:1208–1219, 1975.
3. Brambilla C, DeLena M, Rossi A, et al. Response and survival in advanced breast cancer after two non-cross-resistant combinations. Br Med J 1:801–804, 1976.
4. Tranum B, Hoogstraten B, Kenndey A, et al. Adriamycin in combination for the treatment of breast cancer. A Southwest Oncology Group Study. Cancer 41:2078–2083, 1978.
5. Bull JM, Tormey DC, Li SH, et al. A randomized comparative trial of Adriamycin versus methotrexate in combination drug therapy. Cancer 41:1649–1657 1978.
6. Carbone PP, Bauer M, Band P, et al. Chemotherapy of disseminated breast cancer. Current status and prospects. Cancer 39:2916–2922, 1977.
7. Frei E. Combination cancer therapy: presidential address. Cancer Res 32:2593–2607,1972.
8. Frei E (iii). Effect of dose and schedule on response. *In* Cancer Medicine (Holland JF, and Frei E (iii), eds). Philadelphia, PA, Lea & Febiger, 1973, pp 717–730.
9. Razek A, Valeriote F, and Vietti T. Survival of hematopoietic and leukemia colony-forming cells in vivo following the administration of daunorubicin or Adriamycin. Cancer Res 32:1496–1500, 1972.
10. Canellos GP, DeVita VT, Gold GL, et al. Cyclical combination chemotherapy for advanced breast carcinoma. Br Med J 1:218–220, 1974.
11. Canellos GP, Pocock SJ, Taylor SG (iii), et al. Combination chemotherapy for metastatic breast carcinoma. Prospective comparison of multiple-drug therapy with L-phenylalanine mustard. Cancer 38:1882–1886, 1976.
12. Kaplan EL, and Meier P. Nonparametric estimating from incomplete observation. J Am Stat Assoc 53:457–581, 1958.
13. Emerson JD. Nonparametric confidence intervals for quantiles in the presence of partial right censoring. Sidney Farber Cancer Institute Technical Report 502, 1979.
14. Wilcoxon J. Individual comparisons by ranking methods. Biometrics 1:80–83, 1945.
15. Cox DR. Analysis of binary data. London, Methuen and Co, Ltd., 1970, p 50, 103.
16. Tattersall MHN, Parker LM, Pitman SW, et al. Clinical pharmocology of high-dose methotrexate (NSC-740). Cancer Chemother Rep 6:25–29, 1975.
17. Rossi A, Bonadonna G, Tancini G, et al. Trials of adjuvant chemotherapy in breast cancer. The experience of the Istituto Nazionale Tumori of Milan, Eur J Cancer. In Press.
18. Bonadonna G, and Valagussa P. Dose-response effect after CMF chemotherapy in breast cancer. Proc Am Soc Clin Oncol 21:413, 1980.

Methotrexate Absorption in Children with Acute Lymphoblastic Leukemia [1]

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SUMMARY

The absorption of oral methotrexate (MTX) has been studied in 124 children with acute lymphoblastic leukemia (ALL). Blood levels of MTX were very variable and unpredictable. There was a 20-fold difference between the highest and lowest peak level achieved. Division into two groups has shown that those children who absorb MTX slowly have a worse disease-free survival. It is possible that MTX damages the intestinal mucosa and causes a malabsorption of itself in some children. It is suggested that future protocols for the treatment of ALL should include pharmacokinetic studies, which may help to explain the unexpected relapses of good prognosis patients and lead to the development of more effective treatment.

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Many children with acute lymphoblastic leukemia (ALL) now survive for long periods free of their disease. However, in a portion, the leukemia recurs either during therapy or soon after its cessation. Recent studies (1) have helped to clarify bad prognosis categories, but there remains a group of good prognosis children who relapse. Methotrexate (MTX) is one of the most commonly used antileukemic drugs that form part of the standard maintenance therapy in most treatment protocols (2). In most studies the MTX is given orally and, although it is thought to be well absorbed from the gastrointestinal tract, the rate of absorption and the peak levels reached are known to vary considerably (3,4). The possibility that variations in MTX absorption may explain the unexpected relapse of some children with good prognosis ALL has been investigated in such a group of children.

PATIENTS AND METHODS

We have studied 124 children, 63 boys and 61 girls, between 2 and 11 years of age at diagnosis of their leukemia. They all had a wbc count of less than 20,000/mm^3 at diagnosis, none had a mediastinal mass, and none were of negroid descent. They came from many different centers in the United Kingdom, and were all being treated according to the Medical Research Council UKALL V schedule, which consisted of a common induction regimen of vincristine, prednisone, and asparaginase, then central nervous system prophylaxis with cranial irradiation and intrathecal MTX. This was followed by maintenance therapy with one of three different regimens: intermittent (I), gaps (G), and continuous (C) (fig 1). The dose of MTX recommended to be given on each of the schedules was as follows: I—10 to 12.5 mg/m^2 daily for 5 days; G and C—20 mg/m^2 orally once weekly. Treatment is continued for 2 years from the date of diagnosis. MTX absorption investigations were performed at approximately 6 months or 18 months after diagnosis, and some at both times. The number of patients studied according to the maintenance schedule is shown in table 1. The method of investigation on each occasion was to give the child a normal dose of MTX, to take blood hourly for 5 hours, to separate the plasma, and to send it by post to Guildford for radioimmunoassay (5). No attempt was made to standardize meals. The MTX used in the study was in the form of 2.5 mg tablets commercially available from Lederle Laboratories. The assay cross-reacts with 2,4-diamino-N^{10}-methylpteroic acid by 31% in the absence of MTX. Results were analyzed according to the different schedules of treatment, and the children were also allocated to either a fast or slow absorption group depending on whether or not they

[1] The willing assistance of all the consultants throughout the country who performed the MTX absorption tests is gratefully acknowledged.

[2] On behalf of the Medical Research Council Working Party on Acute Leukaemia in Childhood.

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reached a plasma level of 200 ng/ml (0.44μM) at 1 hour after ingestion of the MTX.

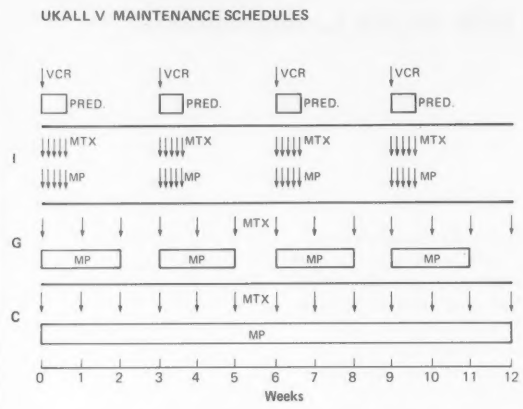

FIGURE 1.—UKALL V maintenance schedules.

TABLE 1.—MTX absorption—UKALL V

Schedule	Number of patients	Number of assays	Repeat assays
I	43	51	8
G	38	45	7
C	43	49	6
Total	124	145	21

RESULTS

There was a wide variation in the rate of absorption and the peak level achieved. The two extremes are shown in figure 2. The 1-hour level varied from 1.7 ng/ml to 895 ng/ml. The mean plasma concentration of MTX according to the maintenance schedule at 6 and 18 months is shown in figures 3 and 4. The I group had the lowest curve on each occasion. The mean peak level achieved according to schedule is shown in figure 5. The I group was again lowest on both occasions. Table 2 shows subdivision of the patients into fast and slow absorption groups. The percentage of children absorbing MTX slowly at 6 months was 31.9%, and at 18 months was 46.1%. Of the patients who had an assay performed at both 6 and 18 months, 11 were in the fast group on both occasions, 7 in the slow group on both occasions, 2 changed from fast to slow, and 1 from slow to fast.

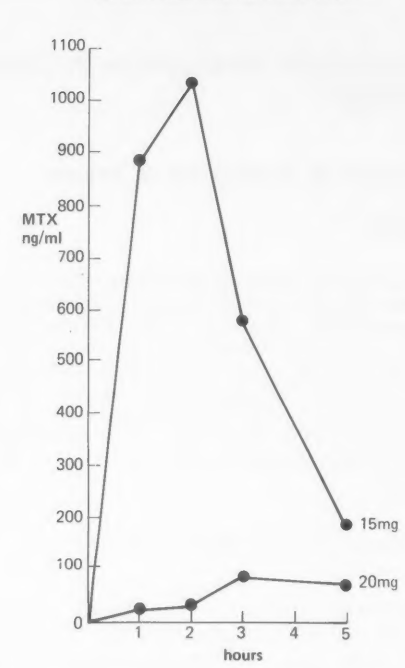

FIGURE 2.—Serum MTX in two patients following single oral dose.

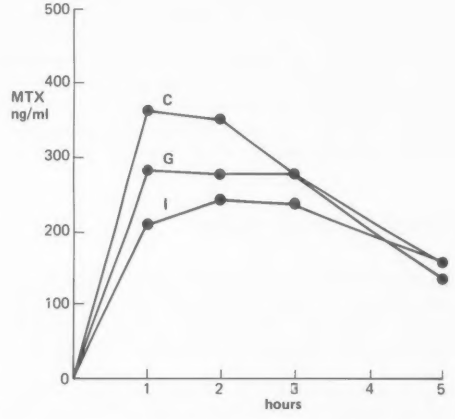

FIGURE 3.—Mean serum MTX according to schedule I, G, or C 6 months after diagnosis.

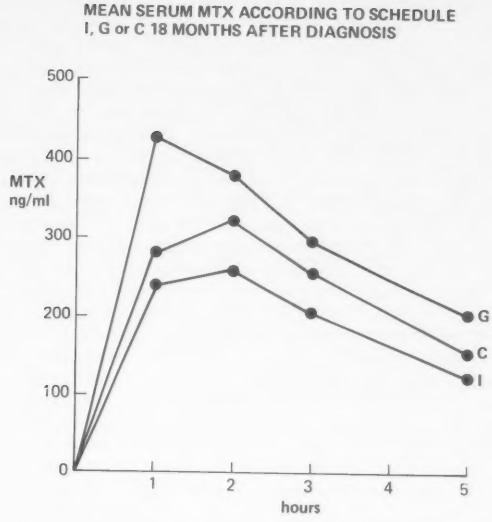

FIGURE 4.—Mean serum MTX according to schedule I, G, or C 18 months after diagnosis.

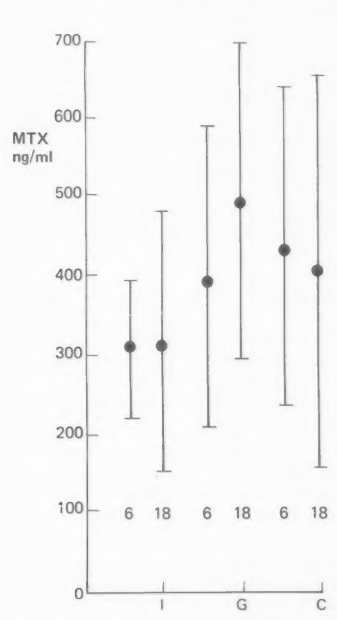

FIGURE 5.—Mean peak MTX according to schedule I, G, or C 6 and 18 months after diagnosis.

TABLE 2.—MTX absorption—UKALL V

Schedule	6 months (n=69)		18 months (n=76)		Total
	Fast	Slow	Fast	Slow	
I	12	8	17	14	51
G	13	8	12	12	45
C	22	6	12	9	49
Total	47	22	41	35	145

Twenty-six of the children have had a relapse of their leukemia. The maintenance schedule and rate of absorption per group are shown in table 3. Of those children who have had a relapse of their leukemia, there is an excess of slow absorbers in the I and G schedules, but not in the C. Overall, 32.7% of the children still in remission were in the slow absorption group compared to 57.7% of the relapse patients. An actuarial disease-free survival curve for the fast and slow groups shows a survival at 40 months of 69% in the slow group and 85% in the fast group (fig 6). Subdivision according to the I and G schedules (fig 7) shows a 91% survival for the I-fast group, 55% for the I-slow, 82% for the G-fast, and 62% for the G-slow. The disease-free survival was 86% for both the fast and slow groups of patients in the C schedule.

TABLE 3.—MTX absorption—UKALL V

Schedule	Remission		Relapse		Total
	Fast	Slow	Fast	Slow	
I	22	11	2	8	43
G	19	9	4	6	38
C	25	12	5	1	43
Total	66	32	11	15	124

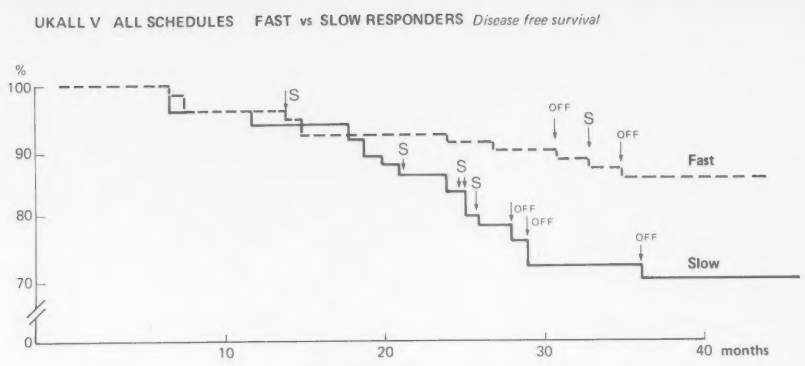

FIGURE 6.—UKALL V all schedules—Fast versus slow responders—disease-free survival.

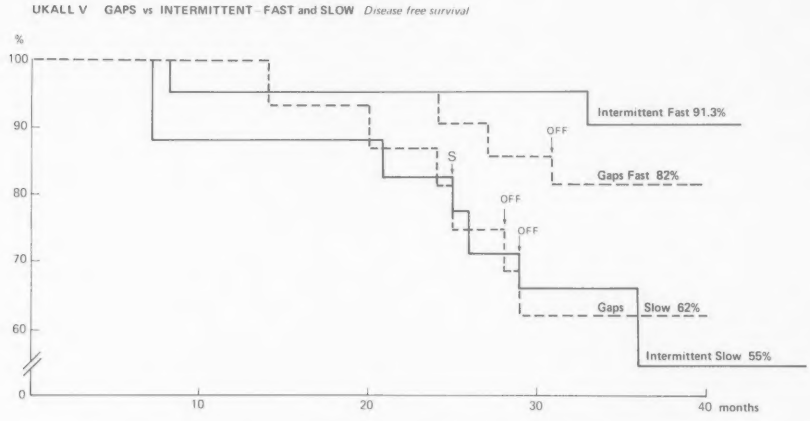

FIGURE 7.—UKALL V gaps versus intermittent—Fast and slow—disease-free survival.

DISCUSSION

The wide variation of plasma levels of MTX previously reported in children given MTX orally (2,3) has been confirmed in the present study, the peak levels varying from 50 to 1,045 ng/ml (0.11 to 2.30 μM)—a 21-fold difference. However, there are other studies (7) claiming that MTX in doses of up to 30 mg/m² is quickly and reliably absorbed from the small intestine and rapidly eliminated via the kidney. In 1961, it was reported that two patterns of absorption could occur, a fast and a slow, and that with continued treatment there was a tendency for those originally absorbing the MTX rapidly to become slow absorbers (3). The present study again suggests that there are two patterns of absorption, but that children are likely to be either fast or slow absorbers and will not change. The majority of children, 18 out of 21, came into the same group at 6 and 18 months, and only 1 changed from slow to fast. However, more of the children who had their test at 18 months (46.1%) were in the slow group than those tested at 6 months (31.9%), perhaps suggesting a trend toward slower absorption with prolonged administration of MTX. There is a difference between the different maintenance schedules in both the rate of absorption and the peak levels achieved. In the I group 43% were in the slow group, 44% in group G, and 30% in group C. The peak level achieved was lowest in group I, but it is possible that some of this difference might be explained by a lower recommended dose of MTX in this group. The main purpose of the present study was to try to determine whether different patterns of absorption of MTX could be related to subsequent outcome in terms of relapse of the leukemia, and it appears that overall those children absorbing MTX slowly have a higher rate of relapse (fig 6). It does not appear that this difference might be due to relapse in

sanctuary sites in the slow group, as there were three sanctuary site relapses in the slow group and two in the fast group. However, the two sanctuary site relapses in the fast group were unusual in that one was an early CNS relapse at 14 months and the other a relapse in the iris at 33 months. Subdivision of the disease-free survival according to maintenance schedule (fig 7) confirms that in the I and G groups the rate of absorption is related to the survival; those children absorbing the MTX slowly have a worse prognosis. There is no difference in group C, but this may be due to a larger number of children in the fast group on this maintenance schedule. The period of followup for the patients in the present study is not yet beyond 46 months, and it may be that, with time, the differences present so far may become more or less apparent. However, it seems that, at least in terms of early relapse both on and off treatment, children who absorb MTX slowly do less well. It is possible that the differences in survival between treatment regimens might be due to the schedules themselves, but even within the schedules there appears to be a difference between the fast and slow absorption groups.

The unpredictability of serum MTX levels may be due to differences in absorption, distribution, or excretion of the drug. MTX is excreted mainly via the kidney, and none of the children in the present study were known to have renal disease. The most likely explanation is that the differences are due to changes in the rate of absorption from the gut. MTX is known to cause damage to the intestinal mucosa in man (8), and in children with ALL malabsorption of xylose it has been related to both the length of administration of the drug and to the spacing of individual doses (9). It is possible, therefore, that MTX produces a malabsorption of itself by damage to intestinal mucosa. Changes in enterohepatic circulation of MTX and the binding to albumin, as well as uptake into tissues, may be other important variables that have not been investigated in the present study.

Up to the present time most treatment regimens for acute lymphoblastic leukemia have been designed and analyzed on a clinical basis. The present study suggests that the pharmacokinetics of the given drugs may be very important to the outcome of a treatment regimen and there is, therefore, need to apply these techniques of investigation of drug handling in subsequent protocols. It is possible that MTX should be given systemically rather than orally to children with ALL because of its unpredictable absorption, and this is to be investigated in a Medical Research Council trial. The application of this knowledge may lead to even better survival prospects for children with ALL.

REFERENCES

1. Chessells JM, Hardisty RM, Rapson NT, et al. Acute lymphoblastic leukaemia in children. Classification and prognosis. Lancet II: 307–309, 1977.
2. Holland JF, and Glidewell O. Oncologists reply–survival expectancy in acute lymphocytic leukaemia. N Engl J Med 287:769–777, 1978.
3. Freeman-Narrod M. The pharmacology of methotrexate. *In* Methotrexate in the Treatment of Cancer (Porter R, and Wiltshaw E, eds). Baltimore, MD, Williams and Wilkins, 1962, pp 17–21.
4. Kearney PJ, Light PA, Preece A, et al. Unpredictable serum levels after oral methotrexate in children with acute lymphoblastic leukaemia. Cancer Chemother Pharmacol 3:117–120, 1979.
5. Aherne GW, Piall EM, and Marks V. Development and application of a radioimmunoassay for methotrexate. Br J Cancer 36:608–617, 1977.
6. Wan SH, Hoffman DH, Azarnoff DL, et al. Effect of route of administration and effusions on methotrexate pharmacokinetics. Cancer Res 34:3487–3491, 1974.
7. Henderson ES, Adamson RH, and Oliverio VT. The metabolic fate of tritiated methotrexate II: absorption and excretion in man. Cancer Res 25:1018–1024, 1965.
8. Trier JS. Morphologic alternations induced by methotrexate in the mucosa of the human proximal intestine. II. Electronmicroscopic observations. Gastroenterology 43:407, 1962.
9. Craft AW, Kay HEM, Lawson DN, et al. Methotrexate induced malabsorption in children with acute lymphoblastic leukaemia. Br Med J II: 1511–1512, 1977.

Asparaginase and Methotrexate Combination Chemotherapy in Relapsed Acute Lymphoblastic Leukemia in Adults [1]

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SUMMARY

We have treated 37 adults with acute lymphoblastic leukemia (ALL), who have relapsed on previous intensive chemotherapy, with a combination of L-asparaginase and methotrexate (MTX) ± ifosfamide. The initial study included 26 patients who received the two-drug combination of L-asparaginase and MTX given sequentially. Of the 26 patients, 15 (58%) achieved complete remission (CR), while 3 patients had a partial remission (PR), resulting in an overall response rate of 69%. The median duration of CR was 17.5 weeks. The median survival of complete responders was 39 weeks compared with 10 weeks for failures (P=0.001). The regimen was generally well tolerated and it was possible to give MTX doses of up to 400 mg/m² with minimal myelosuppression, and severe stomatitis was infrequent. A further study involved the use of this regimen combined with ifosfamide in 11 patients. In this continuing study, there were six CR's (55%) and two PR's, with an overall response rate of 73%. The median duration of CR was 14+ weeks, and the median survival was 40+ weeks. Combination chemotherapy with asparaginase and MTX is effective in inducing remission in adults with ALL in relapse. It should now be considered for inclusion as part of induction therapy of previously untreated adults with ALL.

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The combination of L-asparaginase and methotrexate (MTX) may be either synergistic or antagonistic depending upon the dose and the sequence of drug administration (1–4). L-asparaginase is thought to act primarily by inhibiting protein synthesis, with consequent secondary effects on DNA and RNA synthesis (5). MTX acts primarily by inhibiting DNA synthesis, which is itself dependent on protein synthesis (6). If L-asparaginase is given just before MTX, the cytotoxicity of MTX is considerably decreased. However, if the interval between L-asparaginase administration and that of subsequent MTX is lengthened to allow for recovery of DNA synthesis, there can be enhancement of the therapeutic index of MTX with minimal or no MTX toxicity. These observations were made by Capizzi and others based on preclinical data from the L5178Y mouse leukemia system and on biochemical determinations in human lymphoblasts (3,7). L-asparaginase appears to act as a "rescue" for normal cells from MTX toxicity and may potentiate the antitumor effect of MTX. Why this selective rescue should take place remains unexplained, but clinical experience appears to validate it empirically (8,9).

This paper reports our experience with the use of sequential L-asparaginase and MTX in adult patients with acute lymphoblastic leukemia (ALL), and discusses the use of this type of regimen in combination with other agents.

MATERIALS AND METHODS

L-asparaginase and MTX Regimen

Our first study included 26 patients with previously treated ALL. The median age was 25 years with a range of 16 to 47 years, and the male to female ratio was 19 to 7. All patients received 50 to 80 mg/m² of "unprotected" MTX 3 hours prior to starting the program with L-asparaginase (table 1). L-asparaginase 40,000 iu/m² was then given, followed by MTX 120 mg/m² iv 7 days later. Each dose of MTX was followed by L-asparaginase 24 hours later, and the 2-day

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course of treatment was repeated every 7 to 14 days. The MTX dose was increased to tolerance by increments of 40 mg/m² with each course, while the L-asparaginase dose remained constant, unless abnormal liver function was detected, whereupon the dose was reduced by 50%. Each patient was observed for at least 1 hour for anaphylaxis after each L-asparaginase dose. If anaphylaxis occurred, the patient was switched from *Escherichia coli* L-asparaginase to *Erwinia caratovora* L-asparaginase. The first 15 patients received L-asparaginase at 40,000 iu/m² iv and the next 11 patients on the study received L-asparaginase at 20,000 iu/m² in an attempt to minimize potential L-asparaginase side effects.

TABLE 1.—L-asparaginase (ASP) and MTX treatment program

Day 1	MTX 50 to 80 mg/m² iv
ASP 20/40,000 units/m² iv 3 hours later.	
Day 8	MTX 120 mg/m² iv
Day 9	ASP 20/40,000 units/m² iv
Day 15	MTX } and so on, repeating every 7
Day 16	ASP } to 14 days

Patients were considered to have achieved complete remission (CR) if their bone marrow contained less than 5% leukemic cells and if their peripheral blood contained at least 1,000/mm³ absolute granulocytes and 100,000/mm³ platelets after recovery from hematological toxicity. Patients were considered to have had a partial remission (PR) if their bone marrow contained 6% to 30% leukemic cells with peripheral blood counts as stated above. Patients who achieved CR received maintenance treatment every 2 weeks with the same combination of drugs, the MTX dose being at one level (40 mg/m²) below the toxic dose and the L-asparaginase dose reduced by 50%.

L-asparaginase/Ifosfamide/MTX Regimen (AIM)

Eleven patients have been treated with this combination chemotherapy. There were seven male and four female patients with a median age of 24 years (range, 16 to 40 years). All patients had previously received intensive chemotherapy with regimens containing vincristine, prednisone, and Adriamycin. Eight patients had been previously exposed to cyclophosphamide. On Day 1, all patients received 50 mg/m² of MTX iv 3 hours before 20,000 iu/m² of L-asparaginase iv (table 2). This was followed by ifosfamide 1,000 mg/m²/day iv for the next 3 days. MTX was then repeated on Days 10 and 20, and a further dose of L-asparaginase was given on Day 11. The next cycle of treatment was started on Day 21 with L-asparaginase, followed by 3 days of ifosfamide and so on, repeating every 21 days. The MTX dose was escalated by 40 mg/m² to a maximum of 200 mg/m² or until unacceptable toxicity occurred (eg, stomatitis limiting food intake). The dose of L-asparaginase remained constant, unless there was evidence of abnormal liver function, whereupon the dose was reduced by 50%. Each dose of ifosfamide was given in 2,000 cm³ dextrose solution over 4 hours. Proper hydration was found to be necessary during the administration of ifosfamide. Ideally, all patients should receive at least 3,000 cm³ of fluids per day either orally or, preferably, iv. In addition, patients should be encouraged to empty their bladders frequently and daily urinalysis should be performed during the ifosfamide therapy. The first four patients received ifosfamide at a dose of 1,500 mg/m², and this caused hemorrhagic cystitis in three patients. The dose of ifosfamide was therefore reduced to 1,000 mg/m² during subsequent courses of therapy. For those patients who showed a response of more than 50% reduction in leukemic infiltration, therapy was continued until relapse. When CR was achieved, maintenance therapy was given, with the L-asparaginase dose reduced by 50% and the MTX dose 40 mg/m² below the maximum tolerated dose for that particular patient (table 3). The interval between L-asparaginase and MTX was also lengthened to 14 days, and each cycle of therapy was repeated every 28 days, with the dose of ifosfamide remaining constant.

TABLE 2.—AIM for adult ALL induction therapy

Day 1*	= ASP: 20,000 iu/m², iv
Day 2-4	= Ifosfamide: 1,000 mg/m²/d, iv
Day 10	= MTX: 120 mg/m², iv
Day 11	= ASP: 20,000 iu/m², iv
Day 20	= MTX: 160 mg/m², iv

* MTX 50 mg/m² iv 3 hours after starting regimen (course 1 only).

TABLE 3.—AIM for adult ALL maintenance therapy

Day 1	= ASP: 10,000 iu/m², iv
Day 2-4	= Ifosfamide: 1,000 mg/m²/d, iv
Day 14	= MTX: (established dose)
Day 15	= ASP: 10,000 iu/m², iv
Day 28	= MTX: (established dose)

RESULTS

L-asparaginase and MTX Regimen

Fifteen of the twenty-six patients (58%) achieved CR. Three patients had a PR, resulting in an overall response rate of 69% (18 of 26 patients). The median number of courses required to achieve CR was three and the median treatment days to CR was 30. Of the eight patients who failed to achieve a remission, two died within 10 days of starting therapy. Both patients died from complications of previous treatment, one from cardiomyopathy and the other from septicemia. If only the 24 patients who had received an adequate trial are considered, the overall CR rate was 63% (15 of 26).

The median duration of CR was 17.5 weeks (range, 4 to 49 weeks). The median survival time from the

onset of therapy for complete responders was 39 weeks (range, 15 to 110+weeks) and for failures (excluding the two early deaths) was 10 weeks (range, 3 to 26 weeks, $P=0.001$).

Eleven patients on the study received somewhat lower doses of L-asparaginase (20,000 iu/m^2) in an attempt to minimize L-asparaginase side effects. The CR rate in these patients was 55% (6 of 11), compared with 60% (9 of 15) in the patients who had received doses of 40,000 iu/m^2.

Seven patients had previously received conventional doses of MTX, and the response rate in this group of patients was 71% (5 of 7) compared with 53% (10 of 19) in those who had received no prior MTX. Four patients were MTX-resistant and of these, three achieved CR. Thus, resistance to previous treatment with a conventional dose of MTX did not preclude subsequent response to this regimen.

The toxicities encountered with this regimen have been previously described in a series of 39 patients with acute leukemia (8). The major side effects were due to L-asparaginase. Six patients had acute anaphylactoid reactions and had to be switched from *E. coli* L-asparaginase to *E. caratovora* L-asparaginase, with no hypersensitivity reactions with subsequent courses. A transient rise in liver function test values was seen in 29% of the patients, and 6% of the patients had hypofibrinogenemia. Other side effects noted included nausea and vomiting, diarrhea, abdominal pain, and cachexia. Myelosuppression was minimal with MTX doses up to 280 mg/m^2. Severe stomatitis (defined as multiple mucosal ulcerations limiting food intake) occurred in 11 of 237 courses of treatment. The maximum tolerated dose of MTX was 400 mg/m^2, with a median of 200 mg/m^2. In the majority of patients, stomatitis was the dose-limiting toxicity.

L-asparaginase/Ifosfamide/MTX Regimen (AIM)

Six patients (55%) achieved CR, and the median duration of CR was 14+ weeks with a range of 6 to 56+ weeks. Two patients achieved PR, giving an overall response rate of 73% (8 of 11). The median number of courses to CR was two and the median treatment days to CR was 30. The median survival of complete responders was 40+ weeks with a range of 16+ to 63+ weeks.

Of the three patients who failed to respond, one had a past history of anaphylactoid reaction to *E. coli* L-asparaginase. The patient was given *E. caratovora* L-asparaginase, developed an acute hypersensitivity reaction during the third dose of the drug, and treatment had to be discontinued. The other two patients had severe bone marrow hypoplasia and one of them died on Day 22 with *Klebsiella pneumoniae* septicemia. The third patient died of disseminated aspergillosis and pneumonia within 4 weeks of starting treatment.

Myelosuppression occurred frequently with this regimen, with five patients having severe bone marrow hypoplasia. During induction, the median lowest wbc count was 1,000/mm^3 (range, 0.4 to 3,800/mm^3) and the median lowest platelet count was 74,000/mm^3 (range, 1 to 344,000/mm^3). During maintenance (with MTX doses of 160 to 200 mg/m^2), the median lowest wbc count was 2,400/mm^3 (range, 1.6 to 5,700/mm^3), and the median lowest platelet count was 153,000/mm^3 (range, 50 to 248,000/mm^3). Seven patients developed nausea and vomiting, and stomatitis was observed in four patients. Hematuria occurred in five patients; three of these patients had received ifosfamide at 1,500 mg/m^2 and none had received proper hydration during administration of ifosfamide. Three patients developed pneumonia and septicemia; two of them failed to respond to treatment. Other side effects included fever of unknown origin with neutropenia (two patients), dizziness (two patients), diarrhea (two patients), abdominal pain (one patient), allergic reaction (one patient), and hypoalbuminemia (one patient).

DISCUSSION

Our initial study involved the use of sequential L-asparaginase and MTX, and this regimen was highly effective in reinducing remission in adults with ALL. The CR rate obtained in this study is superior to that achieved with other regimens used in previously treated adult patients (10–12) and is comparable with results of studies on previously untreated patients (13,14). The regimen was generally well tolerated and it was possible to give MTX doses of up to 400 mg/m^2 with minimal myelosuppression. Severe stomatitis was infrequent.

Our next study involved the use of this regimen in combination with ifosfamide. Ifosfamide is an alkylating agent with clinical toxicities very similar to cyclophosphamide, producing nausea, vomiting, alopecia, leukopenia, and hemorrhagic cystitis. Rodriguez et al were able to give ifosfamide in daily doses of 1,200 mg/m^2 during 1 to 2 hour infusions for 5 days with significant reduction of renal and bladder toxicity while preserving its therapeutic efficacy (15).

The AIM regimen, thus far, appears to be effective in reinducing CR in adults with ALL. The objective of adding ifosfamide to the MTX-asparaginase combination was to increase the CR rate and prolong the duration of remission and survival without additional toxicity. A comparison of the AIM program with the sequential L-asparaginase and MTX regimen shows that the two groups of patients are comparable at the start of treatment with respect to age, sex, bone marrow lymphoblasts, wbc count, and platelet count (table 4). The CR rate is also similar (table 5). Both the median duration of CR and survival with AIM are expected to be longer; however, whether the AIM regimen will prove to be more effective than L-asparaginase and MTX alone remains to be seen.

TABLE 4.—ASP/MTX versus AIM for adult ALL—Patient characteristics at start of treatment

	ASP/MTX	AIM
No. of patients	26	11
Age in years (range)	25 (16–47)	24 (16–40)
Male/female	19/7	7/4
Bone marrow lymphoblasts median (range)	68% (30%–99%)	67% (38%–87%)
Wbc count-median (range)	3.4 (0.8–43.0)	5.1 (0.6–40.4)
Platelet count-median (range)	199 (12–552)	248 (11–392)

TABLE 5.—ASP/MTX versus AIM for adult ALL—Results

	ASP/MTX	AIM
No. of patients	26	11
CR's	15 (58%)	6 (55%)
PR's	3 (11%)	2(18%)
No. of courses to CR-median (range).	3 (2–9)	2 (1–3)
Treatment days to CR-median (range).	30 (13–144)	30 (23–49)
Duration of CR-median (range).	17.5 weeks (4–49 weeks).	14+ weeks (6–56+ weeks)
Survival of CR-median (range).	39 weeks (15–110+ weeks).	40+ weeks (16+–63+ weeks)

Encouraged by our results with sequential L-asparaginase and MTX, we have recently introduced this combination as part of the initial treatment of untreated cases of ALL in adults. In this study, six drugs are incorporated for induction therapy, which consists of cyclophosphamide, rubidazone, vincristine, prednisone, asparaginase, and MTX (CROPAM). After the achievement of CR, patients receive one course of cytosine arabinoside and 6-thioguanine and one of cyclophosphamide, rubidazone, vincristine, and prednisone as consolidation therapy. Two bone marrow aspirations and storages are performed during consolidation therapy. Following this, the patient is placed in a protected environment on prophylactic antibiotics and treated with megadose chemotherapy with cyclophosphamide, BCNU, and VP-16, followed by bone marrow rescue. A second course of megadose chemotherapy with autologous bone marrow rescue in the protected environment is repeated after 2 months and, thereafter, all systemic chemotherapy is discontinued. Early results with this protocol are encouraging.

Several other centers are also investigating various L-asparaginase and MTX combinations for the treatment of adult ALL. The Baltimore Cancer Research Center has designed a combination consisting of a 10-day induction course of MTX on Day 1, vincristine on Day 2, and L-asparaginase given 24 hours after MTX administration (16). Dexamethasone was given orally for 10 days. For consolidation therapy, six courses of MTX and L-asparaginase were given every 10 days. This was followed by 12 monthly cytoreduction courses of vincristine, dexamethasone, high-dose MTX, and citrovorum factor rescue. Maintenance therapy with vincristine, dexamethasone, MTX, and 6-mercaptopurine was then given monthly until relapse. In 17 previously untreated patients, the CR rate was 76%, with a median duration of CR of 7.3 months and median survival of 16.7+ months. Nine patients had been previously treated, and in this group of patients the CR rate was 78%, with a median duration of 7.2 months and median survival of 15.7+ months. Thus, L-asparaginase and MTX, combined with vincristine and dexamethasone, is an effective regimen for inducing CR in both previously treated and untreated adults with ALL.

CONCLUSION

It is clear from these studies that combination chemotherapy with sequential L-asparaginase and MTX is successful in inducing remission in adults with ALL. The regimen is easy to administer and generally well tolerated, so it is suitable for use on an outpatient basis, either alone or combined with other agents. We feel that it should now be used not only as a reinduction regimen in previously treated patients, but also as part of induction therapy of previously untreated adults with ALL.

REFERENCES

1. CONNERS T, and JONES M. Antagonism of the anti-tumor effect of asparaginase by methotrexate. Biochem Pharmacol 19:2927, 1970.
2. VADLAMUDI S, KRISHNA B, VENKATA SRV, ET AL. Schedule-dependent therapeutic synergism for L-asparaginase and methotrexate in leukemia (L51784) mice. Cancer Res 33:2014–2019, 1973.
3. CAPIZZI RL. Schedule-dependent synergism and antagonism between methotrexate and asparaginase. Biochem Pharmacol (Suppl 2): 23:151–161, 1974.
4. CHLOPKIEWICZ B, and KOZIOWSKA J. Role of amino acid depletion in combined treatment of neoplastic cells with methotrexate and L-asparaginase. Cancer Res 35:1524–1529, 1975.
5. COONEY DA, and HANDSCHUMACHER RE. L-asparaginase and L-asparagine metabolism. Ann Rev Pharmacol 10:421–440, 1970.
6. BORSA J, and WHITEMORE GF. Cell killing studies on the mode of action of methotrexate in L-cells in vitro. Cancer Res 92:737–744, 1969.
7. CAPIZZI R. Biochemical interaction between asparaginase and methotrexate in leukemia cells. Proc Am Assoc Cancer Res 15:77, 1974.
8. YAP BS, MCCREDIE KB, BENJAMIN RS, ET AL. Refractory acute leukemia in adults treated with sequential colaspase and high dose methotrexate. Br Med J 2:791–793, 1978.
9. LOBEL JS, O'BRIEN RT, MCINTOSH S, ET AL. Methotrexate and asparaginase combination chemotherapy in refractory acute lymphoblastic leukemia of childhood. Cancer 43:1089–1094, 1979.
10. BEARD MEJ, CROWTHER D, GALTON DAG, ET AL. L-asparaginase in treatment of acute leukemia and lymphoma. Br Med J 1:191–195, 1970.
11. RODRIGUEZ V, HART JS, FREIREICH EJ, ET AL. POMP combination chemotherapy of adult acute leukemia. Cancer 32:69–75, 1973.
12. BODEY GP, HEWLETT JS, COLTMAN AC, ET AL. Therapy of adult acute leukemia with daunorubicin and L-asparaginase. Cancer 33:626–630, 1974.
13. GAHRTON G, ENGSTEDT L, FRANZEN S, ET AL. Induction of remission with L-asparaginase, cyclophosphamide, cytosine arabinoside

and prednisone in adult patients with acute leukemia. Cancer 34:472–479, 1974.
14. Lister TA, Whitehouse JMA, Beard MEJ, et al. Combination chemotherapy for acute lymphoblastic leukaemia in adults. Br Med J 1:199–203, 1978.
15. Rodriguez V, Bodey GP, Freireich EJ, et al. Reduction of ifosfamide toxicity using dose fractionation. Cancer Res 36:2945–2948, 1976.
16. Esterhay RJ, Grore WR, and Wiernik PH. Methotrexate, oncovin, L-asparaginase and dexamethasone therapy for adult acute lymphocytic leukemia. Proc Am Assoc Cancer Res and ASCO 20:350, 1979.

Neurologic Sequelae of Methotrexate and Ionizing Radiation: A New Classification[1]

W. Archie Bleyer[2*]

SUMMARY

Therapy for prevention of central nervous system (CNS) leukemia has had a dramatic effect on disease-free survival in children with acute lymphoblastic leukemia (ALL). Now, a majority of children may be in complete remission indefinitely, having completed therapy years ago. Unfortunately, some of these long-term survivors have residual neurologic dysfunction, varying in severity from the not uncommon occurrence of mild intellectual deficit to the fortunately rare instance of debilitating leukoencephalopathy. To help identify inciting factors and ultimately render CNS prophylaxis less neurotoxic, this article attempts to categorize the types of neurotoxicities reported in patients treated with methotrexate (MTX) and ionizing radiation. A variety of clinical syndromes are described and related temporally to these treatment modalities. Analyzed in this way, combinations including CNS irradiation appear to be the most neurotoxic. The safest methods are the single modalities, of which high-dose iv MTX may be the least neurotoxic.

[Cancer Treat Rep 65 (Suppl. 1): 89–98, 1981]

As recently as a decade ago, there were few long-term survivors of childhood acute lymphoblastic leukemia (ALL). Various combinations of systemic chemotherapeutic agents had increased mean survival time to 2 to 3 years, but none of these combinations produced a significant improvement in the 5-year survival rate. It was not until the 1970's, when presymptomatic central nervous system (CNS) therapy was incorporated into the total therapeutic regimen, that the 5-year, relapse-free survival rate improved to the current level of 50% to 60% (1,2). Together with a reduction from 60% to 80% in the incidence of overt CNS leukemia to 5% to 10%, this effect of presymptomatic CNS therapy has been regarded as a "truly sensational breakthrough" in the treatment of childhood ALL (1).

Unfortunately, the dramatic progress has been tempered somewhat by observed and potential toxicities of elective CNS therapy, particularly neurologic sequelae. Both acute and subacute adverse neurologic reactions have been reported and there is an increasing awareness of late CNS effects. Now that a majority of children with ALL may be in complete remission indefinitely, and many have completed therapy years ago, they are being evaluated for deleterious effects of treatment. Some of the long-term survivors have residual neurologic dysfunction, varying in severity from a not uncommon occurrence of mild intellectual dysfunction to a rare instance of debilitating encephalopathy. This article reviews the types of neurotoxicities that have been reported in an attempt to categorize them temporally to their apparent inciting factors.

METHODS

Temporal Relationships

The clinical neurotoxic reactions may be categorized according to time of onset after initiation of CNS therapy. *Acute* reactions occur within hours to days after treatment; *subacute* reactions begin days to weeks later; *delayed* reactions are not noted for several months to years. In general, the acute and subacute forms are either relatively benign or reversible and the delayed toxicities are more serious and usually irreversible.

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Single Versus Combined Modalities

When one of the delayed neurotoxicities, necrotizing leukoencephalopathy, was analyzed for contributing factors, three treatment modalities were found to be involved: cranial irradiation, intrathecal methotrexate (IT MTX), and high-dose iv MTX (HDMTX) (3,4). Of the first 53 patients reported in the literature to have this form of toxicity, 50 had been treated with two or three of these modalities (fig 1). Analysis of the 23 long-term survivors evaluated by Meadows and Evans (5) for residual neurologic sequelae also supported this concept (fig 2). Severe encephalopathy (questionable diagnosis of leukoencephalopathy) was observed in none of 3 patients who were not treated with the above modalities, 1 of 11 who received one modality, and 3 of 9 treated with two or three modalities. Two patients received all three modalities; both had severe encephalopathy. The analysis also suggested that oral MTX has a negligible contribution to delayed neurotoxicity (fig 2). This finding is expected in that the amount of drug absorbed after oral administration and subsequently transported across the blood-brain barrier results in essentially undetectable cerebrospinal fluid (CSF) MTX concentrations.

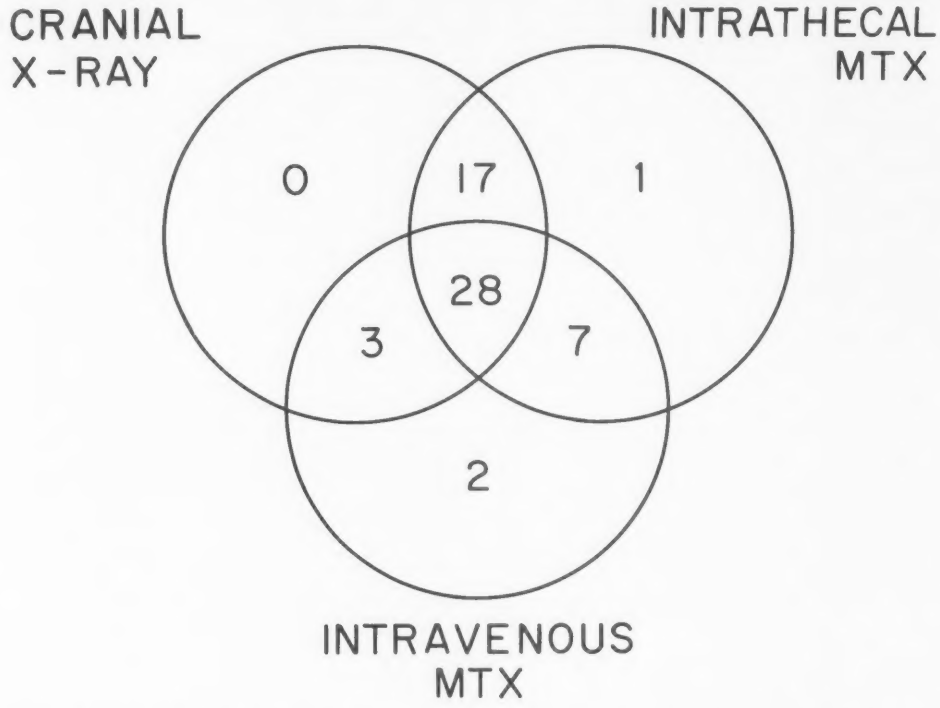

FIGURE 1.—Distribution of the first 53 reported cases of necrotizing leukoencephalopathy according to treatment modality (originally published in reference 4).

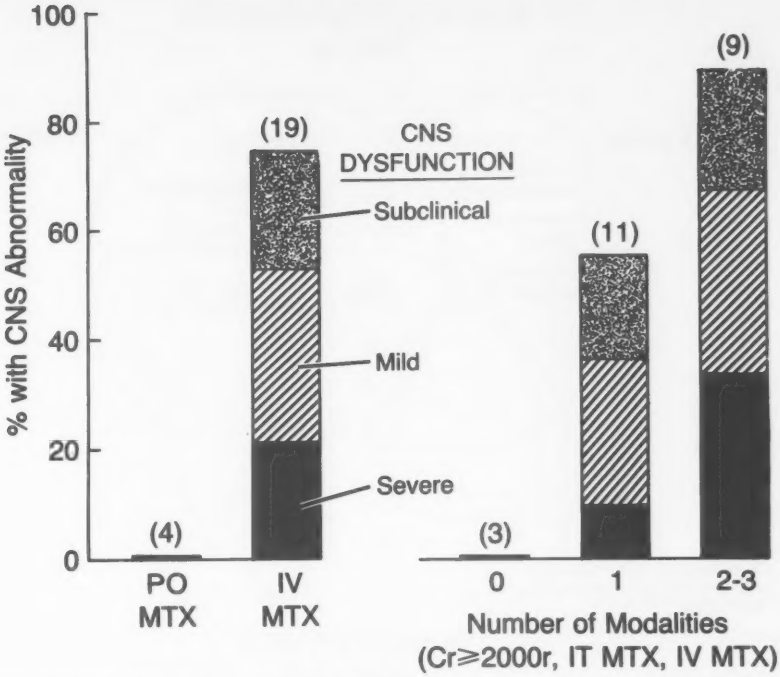

FIGURE 2.—The author's analysis of 23 long-term survivors of childhood ALL studied by Meadows and Evans (5). The histograms on the left compare CNS abnormalities in children treated for more than 2 years with oral (PO) MTX or with iv HDMTX. The histograms on the right compare CNS sequelae according to the number of treatment modalities (see fig 1) administered. The numbers in parentheses indicate the total number of patients in each group.

Hence, the Venn diagram approach shown in figure 1 has proven to be a useful way of illustrating the interrelationships of the factors involved. Also, the contribution of each modality alone can be displayed. For these reasons, the same format was applied in this review to each of the temporally related neurotoxicities associated with MTX and ionizing radiation.

Other Systemic Therapies

Several systemically administered antileukemic drugs have CNS effects, both therapeutic and toxic. Examples are vincristine, prednisone, L-asparaginase, and cytosine arabinoside. The neurotoxicities of these drugs have recently been reviewed (6,7) and are not included in this article. Nonetheless, they probably contribute to the neurotoxicities of prophylactic CNS treatments and the neurotoxic syndromes described in this article should be considered with this point in mind.

SINGLE MODALITIES

CNS Irradiation

Acute neurotoxicity (fig 3).—When administered without concurrent MTX therapy, conventional CNS irradiation is rarely associated with significant adverse neurologic reactions during the course of treatment. Those that do occur are usually mild (nausea, vomiting, headache, drowsiness, anorexia), reversible, and generally avoidable by avoiding high individual radiation fractions (8). Occasionally, exacerbations of preexisting neurologic deficits or indications of increasing intracranial pressure accompany treatment, but these toxicities usually occur in patients with symptomatic or recently excised brain tumors, or with severe CNS leukemia. Most radiotherapists have abandoned the former practice of starting therapy with small doses, 50 to 75 rad, and now start with 180 to 200 rad/fraction (8).

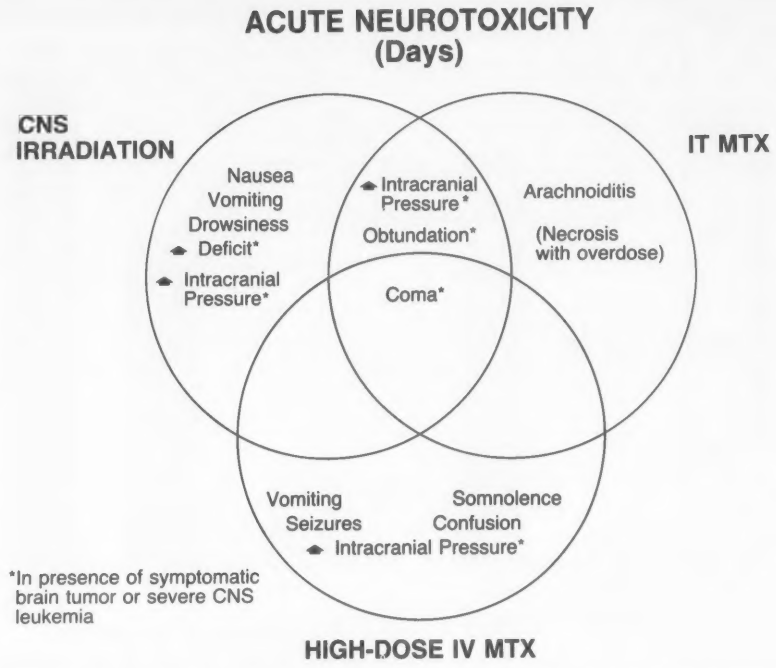

FIGURE 3.—The types of acute neurologic dysfunction associated with CNS irradiation, IT MTX, and iv HDMTX.

Subacute neurotoxicity (fig 4).—More commonly, neurotoxic reactions are subacute, beginning weeks after completing a course of CNS irradiation. If the whole brain is irradiated, even with total doses as low as 1,800 rad, the patient is likely to develop a somnolence syndrome 5 to 7 weeks later. Although characterized primarily by drowsiness, nausea, and malaise, this syndrome may also include anorexia, irritability, fever, dysphasia, ataxia, transient papilledema, and exacerbation of a previous focal deficit (2,7–9). Symptoms may be more severe in children <3 years of age. The EEG nearly always shows a diffuse slowing, and a CSF pleocytosis is occasionally present. The pathophysiology of this reaction is not known, but suspected to involve temporary inhibition of myelin synthesis. The syndrome is completely reversible and symptoms usually abate within 1 to 3 weeks when the radiation dose is 1,800 to 2,400 rad, and longer, eg, 4 to 6 weeks, when it is 4,000 to 5,000 rad. Adrenocorticosteroid therapy may induce a dramatic improvement but treatment with these drugs is generally not needed for this transient toxicity (7). On present evidence somnolence syndrome is benign, but it remains to be established that it is not an indicator of increased risk of latent encephalopathy (2).

Lambert et al (9) describe a patient whose death may have been related to this subacute reaction. This patient, treated for a basal cell carcinoma of the left ear, received 5,000 rad ^{60}Co therapy to a 3 cm depth in 20 fractions over 29 days. Signs of brain stem and cerebellar dysfunction appeared 9 weeks after treatment and the patient expired 4 weeks later.

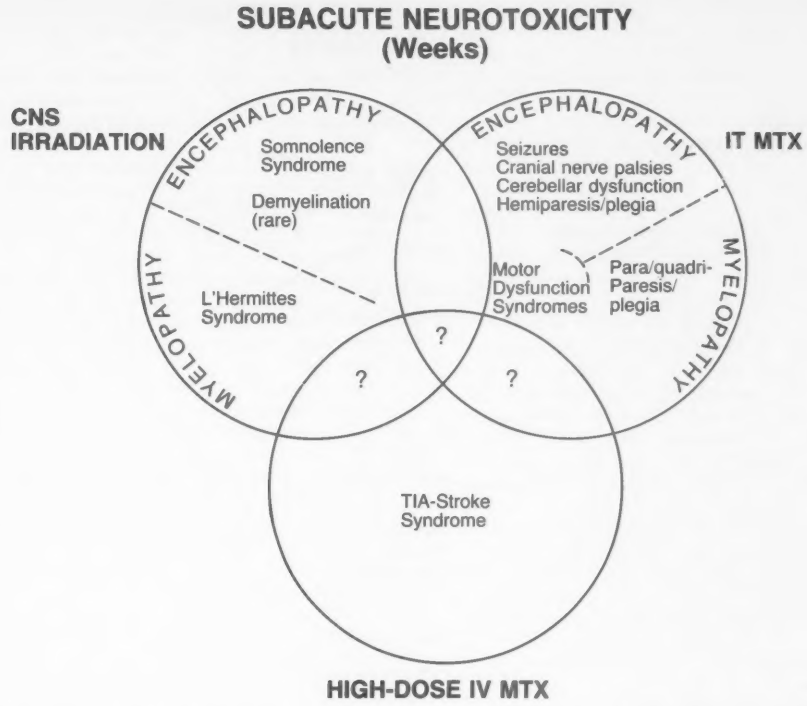

FIGURE 4.—Subacute neurologic dysfunction associated with each of the three treatment modalities. Subacute is defined as beginning weeks after the *start* of CNS irradiation, frequent IT MTX (one to three times weekly), and/or weekly HDMTX.

Autopsy showed a patchy demyelination in both white and gray matter within areas irradiated with 3,000 to 5,000 rad.

If the spinal cord is irradiated, Lhermitte's sign of electrical paresthesia may develop 8 to 16 weeks after therapy. The sign is a subjective sensation of electric shock radiating down the back and over the extremities after neck flexion. As with somnolence syndrome, this toxicity is also transient, resolves within a few months, and is thought to be due to temporary demyelination. Lhermitte's sign may be the spinal cord equivalent of the cerebral somnolence syndrome.

Delayed neurotoxicity (fig 5).—The delayed radiation reaction, cerebral necrosis, is by far the most serious. Beginning insidiously several months to years after treatment and usually diagnosed 1 to 5 years after treatment, it usually progresses to functional impairment or death. Necrosis occurs in areas of the brain exposed to more than 6,000 to 6,500 rad irrespective of whether white or gray matter is involved (10). With conventional radiation techniques, necrosis is a rare event. The relation between age and CNS radiosensitivity is not well established. Doses are ordinarily reduced in the child under 1 to 2 years of age, in whom myelination is incomplete, as suggested by Bloom et al (11). With severe necrosis, a pseudocyst may develop, mimicking tumor recurrence. Cyst formation, when it occurs, usually becomes clinically apparent 2 to 4 years after treatment.

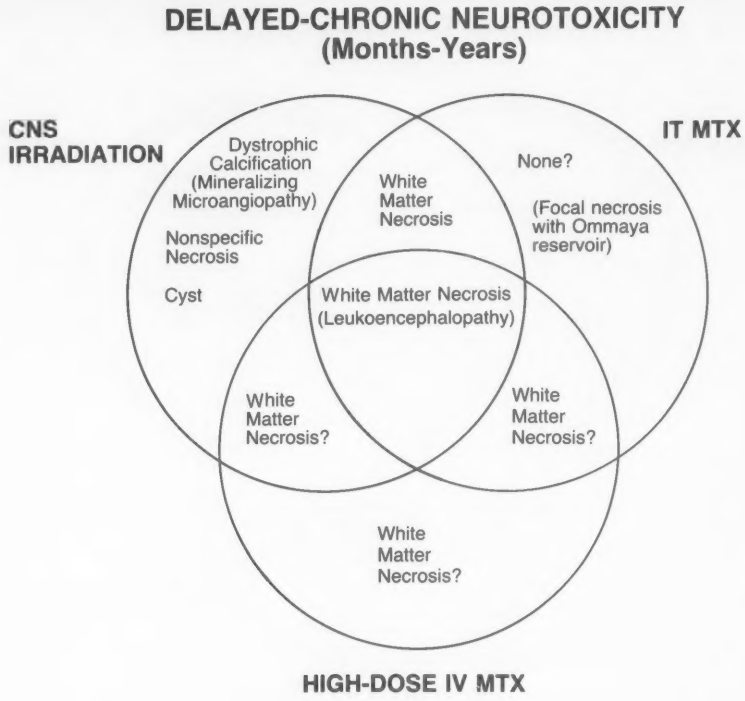

FIGURE 5.—Delayed neurotoxicities associated with the three treatment modalities that become clinically apparent months to years later.

IT MTX

Acute neurotoxicity.—The most common type of MTX neurotoxicity is acute arachnoiditis associated with IT administration (also known as chemical meningitis or acute toxic syndrome). This reaction usually occurs within the first 12 hours following IT administration. It is characterized by headache, nausea, vomiting, meningismus, fever, back pain, dizziness, and other signs of increased intracranial pressure (10). CSF examination often reveals elevated pressure, a granulocytic pleocytosis, and increased protein. Symptoms usually decline within 1 to 2 days but may persist for a week or longer. The frequency with which arachnoiditis has been observed is highly variable and may be influenced by a variety of factors, including the drug diluent and formulation, the dose of MTX given, the schedule of administration and consequent pharmacokinetics, the patient's age, and the presence of overt CNS leukemia (reviewed by Pizzo [7]). In general, clinical arachnoiditis occurs in 5% to 40% of prophylactic IT MTX courses and subclinical arachnoiditis (CSF abnormalities) may occur in a majority of patients. Rarely, the reaction is severe, resulting in nuchal rigidity, increased intracranial pressure, high fever, a massive CSF pleocytosis—a syndrome closely resembling bacterial meningitis. The most severe example shown to the author was a 16-year-old girl with ALL who had a CSF wbc count of 9,700/μl (97% granulocytes) and a CSF protein content of 1,335 mg/dl 9 hours after 12 mg IT MTX in 12 ml Elliott's B solution.[3] All cultures were negative and no antibiotics were given. She improved dramatically on iv dexamethasone and recovered fully within 48 to 72 hours. As in many patients with arachnoiditis (7), her CSF MTX concentrations were elevated.

Subacute neurotoxicity.—A myelopathy or encephalopathy may develop within days to weeks after starting IT MTX therapy. The primary clinical manifestation is motor dysfunction of the spinal cord or brain. This form of toxicity is most frequently encountered in patients receiving frequent IT MTX (two to three times/week). Among 10 reported cases of paraplegia, for example, 9 had 5 to 11 treatments for a total dose of 90 to 192 mg, and 1 had 305 mg in 53 treatments before the onset of paraplegia (12). Symptoms may be

[3] T. Coates, personal communication.

permanent but they are generally reversible after the drug is discontinued or the dosage is reduced. This form of neurotoxicity also appears to be related to the amount and duration of MTX in the CNS (7).

Delayed neurotoxicity.—A leukoencephalopathic form of toxicity has been reported in only one patient treated with IT MTX alone. This patient was a 4-year-old boy with meningeal rhabdomyosarcoma who received a combination of MTX, cytosine arabinoside, and hydrocortisone intraventricularly for 8 consecutive weeks and monthly thereafter (13). After 5 months of treatment, he developed dementia and poor motor function, including dysmetria, myoclonic movements, and generalized clumsiness. CT scan showed nonenhancing hypodensity of the white matter in the centrum ovale and periventricular areas around the frontal horns. There was moderate ventricular dilatation but no calcifications, and other neurologic studies were normal. Within 4 months of discontinuing intraventricular chemotherapy, intellectual function was improved and a repeat CT scan showed less white matter hypodensity. This child could have received prior CNS irradiation when he was treated with 5,000 rad to the site of a primary tumor in the right orbit 1 year prior to the onset of neurologic deterioration.

Allen (6) examined 35 long-term survivors of childhood ALL in whom CNS prophylaxis was limited to IT MTX. None had gross neurologic or psychometric abnormalities, and the CT scan of 10 individuals who had been off all therapy for approximately 3 years revealed no evidence of white matter disease, intracerebral calcifications, or cerebral atrophy. In a study by Obetz et al (14), three of eight children with ALL treated with prophylactic IT MTX alone had late CNS abnormalities. The sequelae were mild: one each with an abnormal EEG, a low psychometric test score, and an abnormality on neurologic examination. None of the eight patients had abnormal CT scans. In the series reported by Meadows and Evans (5), a 5-year-old child with ALL had no clinical, neuropsychologic, or electroencephalographic abnormalities after 3 years' treatment including 2 years of monthly IT MTX for CNS prophylaxis.

Intravenous HDMTX

Acute neurotoxicity.—HDMTX may cause acute somnolence, fatiguability, confusion, disorientation, seizures, or increased intracranial pressure during, or within hours after, iv administration (fig 3). In a pilot study of HDMTX with citrovorum rescue for advanced breast cancer, acute CNS dysfunction was "the most significant toxic effect," 6 of 27 patients developing lethargy, fatiguability, confusion, and disorientation within 24 hours of HDMTX, 2.5 to 7.5 g/m² iv over 6 hours (15). Out of 139 courses of treatment, fatigue and lethargy were noted in 25% and confusion and disorientation in 2% (15). In another series, somnolence and confusion accompanied drug administration in 10% of patients (16). Adults appear to have a higher predilection for HDMTX-induced sedation than children. In view of the rapidity and transiency of acute CNS depression, it is likely that this toxicity is due to a metabolic effect of HDMTX similar to sedative-hypnotic drugs in general. Rarely, seizures occur during bolus injections of HDMTX.[4] In patients predisposed to increased intracranial pressure by the presence of brain metastases or advanced CNS leukemia, HDMTX may precipitate an acute episode of cerebral edema. A rapid reversal of this toxicity may be brought about by systemically administered corticoids. Because this toxicity does not appear to occur in patients with normal brains, the pathogenesis is possibly mediated in part by lysis of tumor cells within the CNS.

Subacute neurotoxicity.—Thus far, the only subacute neurotoxicity that has been associated with HDMTX therapy is an unusual "stroke-like" episode observed by Allen and Rosen (17) in adolescents and young adults with osteosarcoma. In their original report, 4 of 158 patients developed an abrupt onset of a focal cerebral deficit 9 to 13 days after the first, second, or third HDMTX treatment with citrovorum rescue and vincristine (17). All four patients had hemiparesis. Also noted were seizures, aphasia, dysarthria, cranial nerve and gaze palsies, and decreased consciousness. The deficits fluctuated over 2 to 4 days and in two patients the hemiparesis appeared to migrate from one side to the other. In a followup report, a total of eight patients were described to have sustained this syndrome (18). In all patients symptoms abated within a few days and complete neurologic recovery was noted. All neurologic studies, including CT scans on all patients, were normal except for transient, nonspecific EEG changes. Interestingly, this syndrome did not recur after subsequent HDMTX treatments, with as many as 17 treatments administered after recovery from this form of subacute neurotoxicity (17). Also, it has not been reported in patients with other kinds of tumors, implicating osteosarcoma micrometastases in the pathogenesis of this syndrome.

Delayed neurotoxicity.—Four patients have been reported to develop severe encephalopathy after months to years of HDMTX therapy without either IT MTX or CNS irradiation. All four of these patients had extremely high doses of iv MTX, three at weekly intervals for several months, and one over a 5-year period. The latter case is one of the long-term ALL survivors studied by Meadows and Evans (case 1) (5). Diagnosed at age 14 months, this patient's therapy consisted of monthly HDMTX without citrovorum factor rescue, together with intermittent vincristine, prednisone, 6-mercaptopurine, daunorubicin, cytosine arabinoside, cyclophosphamide, and BCNU. After 5 years of treatment with HDMTX, while in complete remission from ALL, the patient developed unremitting seizure activity, spastic quadriplegia, and primitive reflex activity. A biopsy of the right temporal

[4] I. Djerassi, personal communication.

parietal brain showed nonspecific cortical degeneration and white matter gliosis. At the time of report, the leukemia had continued in remission for 2 additional years, but neurologic status had remained unchanged. In the series of patients with osteosarcoma reported by Rosen et al (18), three have been observed to have delayed toxicity resembling leukoencephalopathy. In all three patients, the dose of HDMTX was in excess of 18,000 mg/m², HDMTX was given weekly, and all had nine treatments or more. One patient was a 5-year-old boy who was treated with 10 doses of HDMTX. After his 10th dose he became demented, quadriplegic, unable to speak or understand commands, and semicomatose. A CT scan demonstrated periventricular area white matter hypodensity. Following discontinuation of chemotherapy, the patient gradually improved and by 8 months following the episode was almost completely normal. The remaining two patients had advanced metastatic disease and were treated with very high weekly doses of HDMTX with citrovorum rescue due to the refractoriness of their disease to conventional doses of HDMTX. After nine or more weekly doses, both patients developed a demented vegetative state and then gradually improved after cessation of HDMTX. One eventually died of advanced metastatic osteosarcoma. The other is a long-term survivor who is able to ambulate well and attend school, but still has residual neurologic sequelae.

It is possible to estimate the frequency of these serious delayed reactions with systemic HDMTX alone. In the report by Rosen et al (18), the three patients were drawn from a series of 158 osteosarcoma patients receiving HDMTX with citrovorum rescue (17), indicating an overall incidence of 2%.

COMBINED MODALITIES

CNS Irradiation and IT MTX

Five patients have been described who developed a severe encephalopathy within 3 to 30 hours after the first radiation treatment and one to two doses of IT MTX (19–20). Obtundation, cranial nerve palsies, seizures, ataxia, increased intracranial pressure, and signs of impending herniation were observed, One patient was receiving prophylactic CNS therapy (20); the others had overt CNS leukemia (19). In one patient the reaction was fatal; at autopsy rupture and inflammation of the ependyma were noted in areas of leukemic infiltration (19). The other patients were comatose for 1 to 4 days but recovered. In one patient who had a CT scan 3 months after the acute episode, periventricular hypodensity was noted (20).

A subacute interaction between CNS irradiation and IT MTX has not been clearly discerned. Somnolence syndrome related to brain irradiation is probably increased in frequency or severity when IT MTX is given during the period of radiation. Interestingly, IT MTX-induced arachnoiditis is reduced in patients treated concurrently with cranial irradiation (21).

The delayed sequelae are the most discouraging, particularly in long-term survivors who may have been cured of their disease. These sequelae range in severity from a mild intellectual deficit to a fatal necrotizing leukoencephalopathy. In long-term survivors of childhood ALL, Eiser (22) demonstrated a significantly reduced intelligence among patients treated with cranial irradiation (2,400 rad) and IT MTX, in comparison to children treated with IT MTX alone. The mean intelligence score in 10 children treated with combined therapy was 13 points lower than the mean score in 7 children whose CNS prophylaxis consisted only of IT MTX. Performance skills were affected more than verbal skills, the irradiated group scoring an average of 19 points lower than the nonirradiated group. Two of the irradiated children were 2 years behind on reading skills, in comparison with none of the nonirradiated group. In a noncomparative retrospective study, Verzosa et al (23) showed that about one-third of their patients treated with cranial irradiation, 2,400 rad, plus IT MTX rated below average in concrete-abstract thinking, attention span, motivation, and energy level. Peylan-Ramu et al (24) found CT scan abnormalities in 8 of 14 patients treated with cranial irradiation, 2,400 rad, and IT MTX, including maintenance IT MTX. Four patients had periventricular hypodensity, four had ventricular dilation, three had subarachnoid dilation, and one had intracerebral calcifications. Obetz et al (14) found neuropsychologic abnormalities in 6 of 16 patients treated prophylactically with cranial radiotherapy and IT MTX, 2 with CT abnormalities, 2 with abnormal EEG's, and 2 with an abnormality on neurologic examination or psychometric testing. Histologically, the most frequent finding is white matter necrosis, or leukoencephalopathy (fig 5). This entity has been described in detail elsewhere (3,4,7) and will not be reviewed here.

CNS Irradiation and HDMTX

The clinical impression is that the acute, subacute, and delayed neurotoxic effects of HDMTX are worse in patients previously treated with whole brain irradiation. An insufficient number of patients have been treated with this combination to verify this concept. Three patients in the report by Meadows and Evans (5) were treated with this combination (cases 2, 5, 12). All three had neurologic abnormalities, one of which was severe (5).

IT MTX and HDMTX

Again, little information is available regarding the nature and relative risk of neurotoxicity with this combination. The author's impression is that the combined therapy is only slightly more neurotoxic than either modality alone. In terms of late effects, four patients in the study by Meadows and Evans (5)

had IT MTX and HDMTX (cases 6, 9, 14, 19). One was normal, two had only EEG abnormalities, one had mild psychometric abnormalities, and none had severe impairment. In the study by Obetz et al (14), two of four patients had neurologic dysfunction and both of these were low test scorers on a neuropsychologic battery. The CT scan and EEG's were normal in all four patients. A fatal encephalomyelopathy 3 months after starting IT MTX and low-dose iv MTX has been reported in a child treated with CNS prophylaxis for ALL (25). The CT scan showed only ventriculomegaly. Post-mortem examination revealed extensive subpial necrosis, particularly adjacent to the large subarachnoid cisterns and along most of the surface of the spinal cord. This pattern differs markedly from the central white matter necrosis reported with other combinations.

CNS Irradiation, IT MTX, and HDMTX

As described earlier in this and other reviews (fig 1) (3,4,7), leukoencephalopathy is most likely when all three modalities are used, particularly if the CNS irradiation is administered before or during the systemic MTX therapy. Aur et al (26), for example, observed crippling encephalopathy in 9 of 20 children treated with the standard CNS prophylaxis of cranial irradiation, 2,400 rad, and IT MTX twice weekly for five doses, followed by weekly iv MTX, 40 to 80 mg/m^2. McIntosh et al (27) studied 23 children treated with standard CNS prophylaxis followed by iv MTX, cyclophosphamide, and cytosine arabinoside every other week. The MTX dose was started at 40 mg/m^2 and escalated to maximally tolerated levels, ranging from 114 to 448 mg/m^2. Between the 10th and 18th months of maintenance therapy, during which maximally tolerated dosage of MTX was reached, 12 patients developed vague symptoms of intermittent limping and incoordination. Seizures, followed by varying degrees of hyperkinesis, ataxia, or subnormal perceptual-motor skills, developed in five of these children. Three school-aged youngsters have learning disability from perceptual-motor handicaps. Psychological testing delineated significant abnormalities in gross or fine motor, perceptual, language, or behavioral function in 8 of 11 symptomatic patients tested. McIntosh and her colleagues have also reported intracranial calcifications in 10 of 25 patients treated with all three modalities (28). The risk of leukoencephalopathy appears to be related to the cumulative dose of MTX administered, as first proposed by Price (3). This observation is supported by the report of McIntosh et al (28), in which calcifications were found in 9 of 14 patients exposed to a cumulative iv dose of MTX > 4.5 g/m^2 and in only 1 of 11 patients treated over a comparable period of time with less than this amount.

DISCUSSION

When assembled in Venn diagram fashion, the four potential interactions of CNS irradiation, IT MTX, and HDMTX are signified by the overlapping portions of the circles. By using a different Venn diagram for acute, subacute, and delayed reactions, 12 possible interactions are displayed (figs 3, 5). From the data reported in the literature thus far, only one interaction appears to result in decreased neurotoxicity. The single exception is the reduction in acute arachnoiditis when IT MTX is given during cranial irradiation (21). The biologic basis for this interaction has been attributed to inhibition by ionizing radiation of the acute inflammatory response to IT MTX injections (21). Other explanations are that ionizing radiation increases the clearance of MTX from the CNS, alters the distribution of MTX in the CNS such that the meninges are exposed to less MTX, or increases extracellular CNS concentrations of MTX antagonists such as 5-methyltetrahydrofolate or thymidine.

All of the other possible combinations have been shown, or can be predicted, to result in increased neurotoxicity. There are basically three likely mechanisms for these additive, and possibly synergistic, reactions: (a) ionizing radiation and MTX have overlapping mechanisms of cytotoxicity (eg, both inhibit macromolecular synthesis and DNA repair); (b) MTX is a radiosensitizer (29) and can increase the sensitivity of brain cells to ionizing radiation; (c) CNS irradiation alters the distribution kinetics of MTX in the CNS such that certain areas of the brain accumulate increased amounts of antifolate. The latter mechanism has several possibilities: (a) radiation may increase the permeability of the blood-brain barrier, allowing more systemically administered MTX to enter the brain (30); (b) by acting on the arachnoid granulation of the choroid plexus, radiation may slow the turnover of CSF, decreasing the clearance of MTX from the CNS; (c) ionizing radiation may disrupt the ependymal barrier, allowing more MTX to diffuse from the ventricles into the central white matter; and (d) individual brain cells may accumulate more MTX because of radiation-induced changes at the cellular level.

CONCLUSION

It is not known which of the many possible mechanisms are operative, or predominate, in the etiology of iatrogenic neurotoxicity. What is apparent is that, with the one exception described above, the risk and severity of neurotoxicity are directly proportional to the number of therapeutic modalities used. Three is worse than two, and two is worse than one. This review also suggests that combinations including CNS irradiation appear to be the most neurotoxic. The least neurotoxic combination of two modalities

appears to be IT MTX and HDMTX. Thus far, HDMTX appears to be the safest single modality, in terms of acute, subacute, and delayed neurotoxicity. The analysis presented here further suggests that, if CNS irradiation must be combined with MTX therapy, the least neurotoxic approach would be to administer these modalities in sequence, IT MTX or HDMTX followed by CNS irradiation. MTX given during or after CNS irradiation is much more likely to produce severe neurologic sequelae.

REFERENCES

1. Pochedly C. Prophylactic CNS therapy in childhood acute leukemia. Review of methods used. Am J Pediatr Hematol Oncol 1:119–126, 1979.
2. Jenkin DT. Radiation in the treatment of meningeal leukemia. Am J Pediatr Hematol Oncol 1:49–58, 1979.
3. Price RA. Histopathology of CNS leukemia and complications of therapy. Am J Pediatr Hematol Oncol 1:21–30, 1979.
4. Bleyer WA. Current status of intrathecal chemotherapy for human meningeal neoplasms. Natl Cancer Inst Monogr 46:171–178, 1977.
5. Meadows AT, and Evans AE. Effects of chemotherapy on the CNS: a study of parenteral MTX in long term survivors of leukemia and lymphoma in childhood. Cancer 37:1079–1085, 1976.
6. Allen JC. The effects of cancer therapy on the nervous system. J Pediatr 93:903–909, 1978.
7. Pizzo PA, Poplack DG, and Bleyer WA. Neurotoxicities of current leukemia therapy. Am J Pediatr Hematol Oncol 1:127–140, 1979.
8. Sheline GE. Radiation therapy of primary brain tumors. Semin Oncol 2:29–42, 1975.
9. Lampert P, Tom MI, and Rider WD. Disseminated demyelination of the brain following ^{60}Co (Gamma) radiation. Arch Pathol 68:322–330, 1959.
10. Mikhael MA. Radiation necrosis of the brain: correlation between computed tomography and dose of radiation. J Comput Assist Tomogr 3:241–249, 1979.
11. Bloom HJG, Wallace ENK, and Henk JM. The treatment and prognosis of medullo-blastoma in children. Am J Roentgenol Rad Ther Nucl Med 105:43–62, 1969.
12. Gagliano G, and Costanzi J. Paraplegia following intrathecal methotrexate: report of a case and review of the literature. Cancer 37:1663–1668, 1976.
13. Fusner J, Poplack DG, Pizzo PA, et al. Leukoencephalopathy following chemotherapy for rhabdomyosarcoma; reversibility of cerebral changes demonstrated by computed tomography. J Pediatr 91:77–79, 1977.
14. Obetz SW, Smithson WA, Groove RV, et al. Neuropsychological followup study of central nervous system function in children with acute lymphocytic leukemia. Proc Am Assoc Cancer Res and ASCO 20:342, 1979.
15. Yap H, Blumenschein GR, Yap BS, et al. High-dose methotrexate for advanced breast cancer. Cancer Treat Rep 63:757–761, 1979.
16. Abelson HT. Methotrexate and central nervous system toxicity. Cancer Treat Rep 62:1999–2001, 1978.
17. Allen JC, and Rosen G. Transient cerebral dysfunction following chemotherapy for osteogenic sarcoma. Ann Neurol 3:441–444, 1978.
18. Rosen G, Marcove RC, Caparros B, et al. Primary osteogenic sarcoma. Cancer 43:2163–2177, 1979.
19. Oliff A, Bleyer WA, Poplack DG. Acute encephalopathy after initiation of cranial irradiation for meningeal leukemia. Lancet 2:13–15, 1978.
20. Casteels-van Daele M, and van de Casseye W. Acute encephalopathy after initiation of cranial irradiation for meningeal leukemia. Lancet 2:834–835, 1978.
21. Geiser CF, Bishop Y, Jaffe N, et al. Adverse effects of intrathecal methotrexate in children with acute leukemia in remission. Blood 45:189–195, 1975.
22. Eiser C. Intellectual abilities among survivors of childhood leukaemia as a function of CNS irradiation. Arch Dis Child 53:391–395, 1978.
23. Verzosa MS, Aur RJA, Simon JV, et al. Five years after central nervous system irradiation of children with leukemia. Int J Rad Oncol Biol Phys 1:209–215, 1976.
24. Peylan-Ramu N, Poplack DG, Blei CL, et al. Computer assisted tomography in methotrexate encephalopathy. J Comput Assist Tomogr 1:216–221, 1977.
25. Skullerud K, and Halvorsen K. Encephalomyelopathy following intrathecal methotrexate treatment in a child with acute leukemia. Cancer 42:1211–1215, 1978.
26. Aur R, Veroza M, Hustu O, et al. Leukeoencephalopathy during complete remission in children with acute lymphocytic leukemia receiving methotrexate. Proc Am Assoc Cancer Res 16:92, 1975.
27. McIntosh S, Klatskin EH, O'Brien RT, et al. Chronic neurologic disturbance in childhood leukemia. Cancer 37:853–857, 1976.
28. McIntosh S, Fischer DB, Rothman S, et al. Intracranial calcifications in childhood leukemia. J Pediatr 91:909–913, 1977.
29. Berry RJ, and Asquith JC. Cell cycle-dependent and hypoxic radiosensitizers. *In* Advances in Chemical Radiosensitization. Vienna, International Atomic Energy Agency, 1977, pp 25–36.
30. Griffin TW, Rasey JS, and Bleyer WA. The effect of photon irradiation on blood-brain barrier permeability to methotrexate in mice. Cancer 40:1109–1111, 1977.

High-Dose Methotrexate in Osteogenic Sacroma Adjuvant Chemotherapy and Limb Salvage Results [1]

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SUMMARY

This communication provides a description and update of the three adjuvant regimens utilized at the Sidney Farber Cancer Institute. The disease-free survivals are: Study I—42% at 4 years; Study II—58% at 3 years; and Study III—currently 78%. In the limb salvage experience, among the evaluable patients, 6 of 12 treated with preoperative chemotherapy are disease free (median 32 months) and 12 of 14 (86%) treated without preoperative chemotherapy are disease free (median 13 months).

[Cancer Treat Rep 65 (Suppl. 1):99–106, 1981]

High-dose methotrexate (MTX) with citrovorum factor (CF) rescue (MTX-CF) forms the basis of several therapeutic regimens for the treatment of osteogenic sarcoma. Its application as adjuvant therapy in metastatic disease, and as preoperative therapy for limb salvage, has been described in several communications (1–5). The purpose of this report is to update the results of the three adjuvant chemotherapy regimens and limb salvage procedures utilized at the Sidney Farber Cancer Institute.

ADJUVANT STUDIES

The initial treatment regimen consisted of vincristine (V), followed after one-half hour by MTX administered over 6 hours. Two hours later, CF rescue was started. The doses of MTX were escalated from 3 to 7.5 g/m², and are now standardized at 7.5 g/m². The regimen was administered at 2- to 3-week intervals (fig 1). Details of MTX administration and CF rescue have been previously described (5).

The above regimen produced a 35% response rate in metastatic disease and was adopted as adjuvant therapy at the Sidney Farber Cancer Institute in 1972 (1,2). The investigation was designated Study I. Treatment was administered at 21-day intervals for 18 to 24 months. The second regimen included Adriamycin (ADR) (Study II), and was initiated in 1974 and terminated in 1976. ADR was administered at a dose of 75 mg/m² 7 days after each V-MTX-CF course, and the interval between courses was extended to 28 days. After the ADR dose had accumulated to 450 mg/m², the triweekly V-MTX-CF schedule was reinstated. Treatment was administered for 18 months (fig 2).

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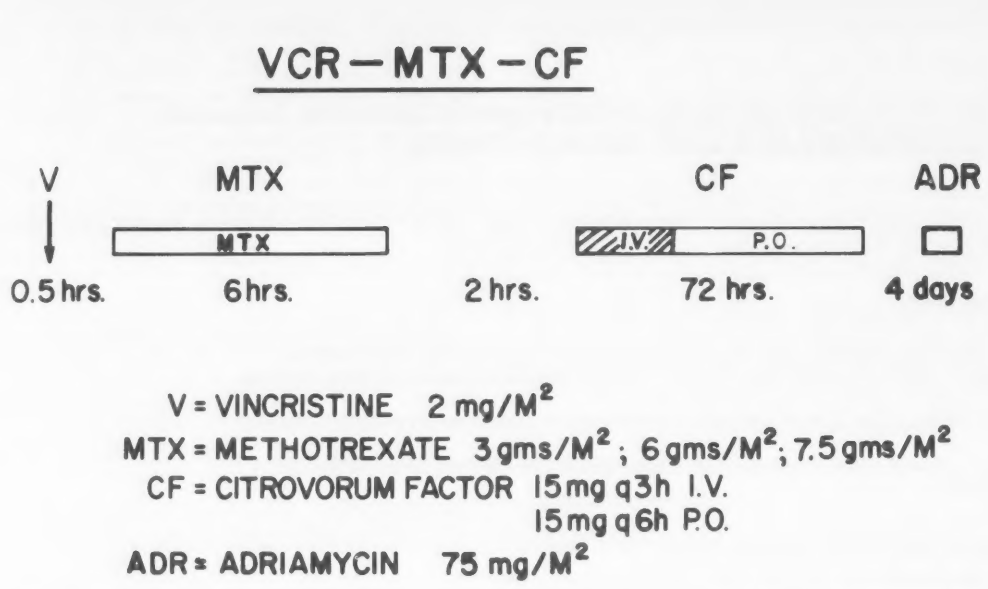


FIGURE 1.—Basic regimen (Study I). V = vincristine 2 mg/m² (maximum 2 mg); MTX = methotrexate 3, 6, and 7.5 g/m²; CF = citrovorum factor 15 mg q 3 hours iv and 15 mg q 6 hours po. The interval between the first and second doses of MTX is 2 weeks and between the second and subsequent doses is 3 weeks. Adjustments in schedules were made by incorporating Adriamycin (ADR) and weekly treatments (see fig 2).

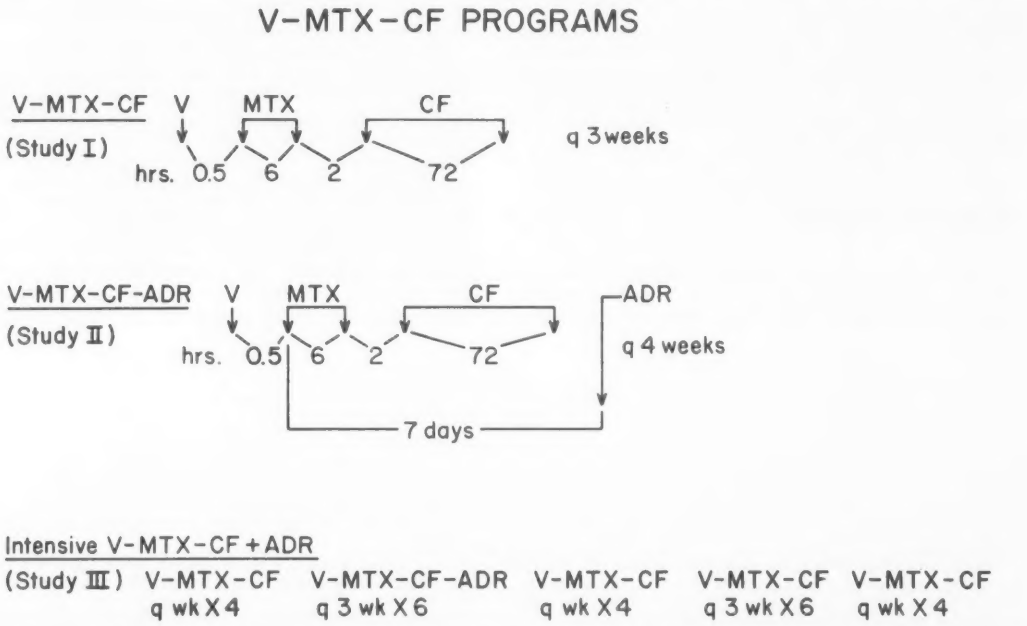


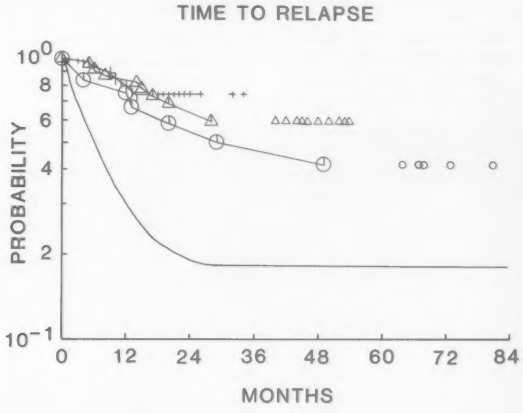
FIGURE 2.—Study I: Basic regimen (fig. 1). Study II: ADR (75 mg/m²) administered 7 days after V-MTX-CF. Maximum cumulative ADR dose 450 mg/m². V-MTX-CF-ADR administered q 4 weeks. Study III: ADR (75 mg/m²) administered 6 days after V-MTX-CF. V-MTX-CF-ADR administered q 3 weeks. Three V-MTX-CF q week × four courses also employed.

Since V-MTX-CF was rarely followed by myelosuppression, a study to determine the feasibility and therapeutic efficacy of its administration on a weekly basis was undertaken. An 88% response rate was achieved in patients with pulmonary metastases or primary tumors not previously exposed to MTX (4). This high initial response rate fell to 48% as greater numbers of patients were treated; however, it provided the impetus for incorporating the weekly MTX regimen into a third adjuvant program (5). This was designated Study III and was initiated in 1976. Therapy was more intensive and the duration of treatment was shortened to 12 months. Four weekly V-MTX-CF courses were administered during the 1st, 6th, and 12th months of treatment. In the interval between the first and second courses, V-MTX-CF-ADR were administered once every 3 weeks. The dose of ADR was 75 mg/m² as in Study II, but the interval between V-MTX-CF and ADR was reduced from 7 to 6 days. Treatment was initiated 2 to 3 weeks after ablation of the primary tumor (fig 2).

Pretreatment evaluation for each study consisted of a hemogram, liver function studies, BUN, serum creatinine, and creatinine clearance. A hemogram, serum MTX, BUN, and creatinine were obtained daily during treatment, and liver function studies were performed at the completion of each course. Treatment was withheld or delayed in a variety of situations, including abnormal creatinine clearance, clinical jaundice, severe infection, stomatitis, dehydration, and myelosuppression (leukocyte count under 2,000/ml³ or platelet count under 75,000/ml³). Throughout the course of therapy, adequate hydration was maintained. Liver function studies and a hemogram were also obtained prior to each ADR administration.

The results of the three adjuvant studies are plotted as Kaplan-Meier survival curves (6) (fig 3). In Study I, 7 of 12 patients with classical osteosarcoma developed pulmonary metastases. Five remain free of disease for 4+ to 5+ years (4-year disease-free survival 42%). In Study II, 9 of 22 patients developed pulmonary metastases. Thirteen remain free of disease for 3+ to 5+ years (3-year disease-free survival 58%). In Study III, 6 of 30 patients have developed pulmonary metastases. Twenty-four patients remain free of disease for 4+ to 39+ months with a median followup of 19 months. In contrast to the above, 50% of historical control patients developed pulmonary metastases by 6 months, and 80% by 12 months (3).

Over 3,000 V-MTX-CF courses have been administered. The regimen was found to be relatively safe with the major toxicities being nausea, myelosuppression, stomatitis, convulsions, and renal impairment (7).



STUDY	FREE	RELAP	TOTAL	MEDIAN
○ STUDY I	5	7	12	29.0
△ STUDY II	13	9	22	UNDEF
+ STUDY III	24	6	30	UNDEF

FIGURE 3.—Incidence of pulmonary metastases. The lowest curve represents the incidence of pulmonary metastases in 78 control patients (3).

Limb Salvage

The successes achieved with adjuvant chemotherapy encouraged attempts at limb salvage (8,9). Eligibility for the procedure included all patients with resectable lower extremity tumors who had attained maximal or near maximal linear growth. In contrast all patients with upper extremity tumors received favorable consideration. Each patient's expectations for subsequent strenuous physical activity were considered a major factor in the recommendation of a limb salvage procedure versus amputation.

Initially, limb salvage operations involved construction of a custom-made prosthesis that required several weeks for manufacture. During the delay, preoperative chemotherapy was administered in an attempt to destroy the primary tumor and ablate micrometastases. Treatment regimens consisted of V-MTX-CF alone or in combination with intraarterial ADR. Varying degrees of tumor destruction attributable to chemotherapy were noted in 9 of 13 patients (4,5,9). Local en bloc resection was then performed. Postoperatively, adjuvant therapy with V-MTX-CF-ADR (Studies II or III) was administered.

Attempts have been made to correlate pre- and postchemotherapy angiograms with clinical and pathological responses. Clinical responses were based on reduction in tumor size and improvement in pain. Pathologic studies involved macroscopic and microscopic examination of an average of one tissue block/cm of the maximum tumor diameter. Chemotherapy effect was determined on the basis of tumor destruction and vascular and fibroblastic proliferation. The degree of tumor destruction was arbitrarily assigned a percentage that acknowledged the probability that intrinsic destruction was usually less than 30% and very rarely exceeded 50%.

Angiographic responses were classified as positive, mixed, and negative (table 1). A positive response (ie, reduction and/or disappearance of tumor neovascularity) (fig 4) was observed in four patients. The findings were consistent with over 70% tumor destruction and generally correlated with improvements in clinical parameters (figs 5 and 6). One patient with a hypovascular tumor did not demonstrate any substantial angiographic changes; however he achieved over 70% tumor destruction (fig 7).

TABLE 1.—Angiographic responses

Patients	No. V-MTX-CF courses	Intra-arterial ADR infusion (patients)	Angiography	Tumor destruction
4	6 to 8	2	Positive	70% to 100%
1	6	–	No change	Over 70%
4	6 to 8	1	Mixed	50% to 60%
*3	2 to 4	1	Negative	Under 30%

* Post-treatment angiogram not performed in one patient.

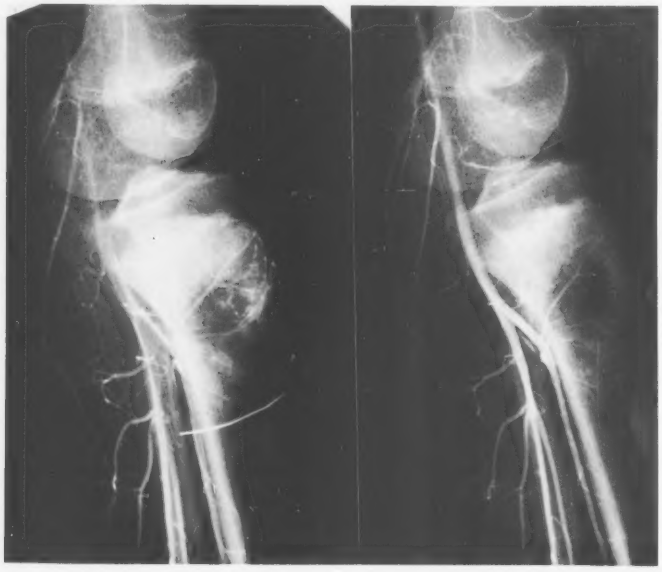


FIGURE 4.—Left: Pretreatment angiogram demonstrating tumor blush and neovascularity in a patient with an osteogenic sarcoma of the proximal tibia. Right: Following 8 weekly V-MTX-CF courses there has been a marked reduction in tumor neovascularity.

FIGURE 5.—Left: Swelling of limb due to tumor at commencement of treatment. Right: Reduction of tumor mass after 8 weekly V-MTX-CF courses. This was accompanied by complete disappearance of pain.

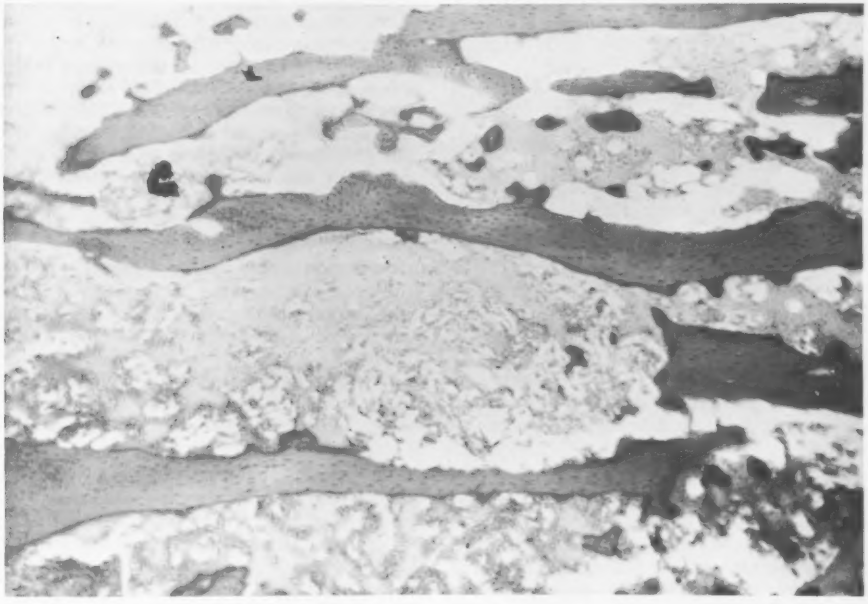


FIGURE 6.—Necrotic distorted tumor cells and debris in osteoid matrix following treatment of tumor described in figures 4 and 5. Over 70% tumor destruction was achieved.

FIGURE 7.—Left: Hypovascular tumor with limited neovascularity (subtraction view). Right: Angiogram obtained after 6 weekly V-MTX-CF courses. The changes were considered minimal. However, tumor destruction was assessed at over 70%.

A mixed response (ie, disappearance of tumor neovascularity in some areas and persistence or increase in the findings in others) (fig 8) was observed in four patients and was consistent with 50% to 60% tumor destruction. The clinical parameters varied. Generally there was some reduction in pain with little change in tumor size.

A negative response (ie, an increase in tumor neovascularity, pain, and size) was observed in three patients (a post-treatment angiogram was not performed in the fourth patient). Pathologically the degree of tumor destruction was assessed as under 50%.

In general, decreased tumor neovascularity correlated with clinical findings and more extensive tumor destruction as judged by pathological examination. However, this correlation was not precise. Angiographic responses, therefore, must be interpreted with caution and at present should be employed only as ancillary investigations to determine chemotherapy effect.

The current status of patients treated with preoperative chemotherapy is outlined in table 2. One patient with a tibial lesion who prematurely terminated adjuvant therapy is considered inevaluable although he remains disease free for over 28 months. Of the remaining 12 study patients, 6 (50%) are continuously disease free with a median followup of 32 months.

Subsequently, immediate en bloc resections became feasible since a supply of prefabricated endoprostheses were acquired. Preliminary results of the treatment of these patients have been reported (5). To date, 18 patients have undergone a variety of limb salvage procedures without preoperative chemotherapy (table 2). Of these, 14 have good function of the resected extremity. One with a distal femur lesion has a poor functional result and three have required subsequent amputation. Good function is defined as the ability to use the resected extremity for normal activities, but strenuous activities are often limited.

This group of 18 patients was also analyzed with respect to disease-free survival. For this purpose, two patients with multiple bony-site disease at presentation, one in whom the diagnosis was later revised to mesenchymal sarcoma, and one who failed to complete adjuvant chemotherapy, are excluded. Of the remaining 14 study patients, 12 (86%) remain continuously disease free for 3 to 25 months, with a median followup of 13 months. Of the two patients who developed pulmonary metastases, one has died, and the other remains disease free 5 months after surgical removal of a single pulmonary lesion.

Of the four patients excluded, one with multiple bony-site disease has progression at a nonresectable site; the other remains continuously disease free for 16 months. The patient with a mesenchymal sarcoma developed a single pulmonary metastasis at 14 months and is now disease free 3 months after thoracotomy. The patient with the humeral lesion who was unable to complete adjuvant therapy has been continuously disease free for 25+ months (table 2).

FIGURE 8.–Left: Staining and tumor neovascularity in a patient with a distal femur lesion prior to treatment (subtraction view). Right: The post-therapy angiogram demonstrates disappearance of tumor neovascularity on one osseous border. However, on the opposite side neovascularity persists with tumor staining. This was classified as a mixed response with 50% to 60% tumor destruction.

DISCUSSION

The conceptual development and design of the adjuvant studies were based on pharmacologic and kinetic principles (10). Thus, an attempt was made to destroy pulmonary micrometastases with sustained and repeated courses of chemotherapy, ie, agents with different mechanisms of action administered at optimally determined doses and schedules. The approach proved feasible in view of the predominantly nonmyelosuppressive nature of the V-MTX-CF regimen (4). To enhance the degree of cytoreduction, successive protocols were designed to be increasingly more aggressive.

This update of the results provides continued support for previous assertions that affective adjuvant chemotherapy has changed the course of this once almost uniformly fatal disease. The trend emerging from the three generations of protocols is consistent with the results obtained by others using aggressive combinations of MTX and ADR (11).

The results of our limb salvage experience demonstrate that, for selected patients, these procedures produce an extremity that is both functionally and cosmetically acceptable. More importantly, although the followup is relatively short, the disease-free survival of these patients appears to be as good as those treated by amputation.

The results reported herein indicate that major advances in the treatment of osteogenic sarcoma have been attained. Pulmonary micrometastases appear to have been eradicated in the majority of patients and improvement in the quality of life has been achieved through limb salvage. These studies foster cautious enthusiasm and indicate the need for additional exploration to enhance the therapeutic efficacy of both adjuvant chemotherapy and surgical management.

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TABLE 2.—Limb Salvage Treatment—Results with preoperative chemotherapy and immediate surgery

Treatment	Total number of patients	Site of primary tumor				Number of evaluable patients on adjuvant chemotherapy	Number with pulmonary metastases	Number alive	Continuously disease-free (%) (study patients)	Median followup (months)
		Humerus	Femur	Tibia	Fibula					
Preoperative chemotherapy	13	1	*7 (2)	**4 (2)	1	12	6	6	50	32
No preoperative chemotherapy	18	[d]3	++9 (1)	[d]3 (1)	++4 (1)	14	2	14	86	13

*Numbers in parentheses denote patients undergoing delayed amputation for infection or ulceration.
**One patient refused continuation of adjuvant therapy. Alive and disease free over 28 months.
++One patient in each group with multiple disease.
[d] One patient in each group not on study. Patient with primary humeral lesion continously disease free for 25+ months. Patient with primary tibial lesion developed single pulmonary metastasis at 14 months that was surgically resected.

REFERENCES

1. JAFFE N. Recent advances in the chemotherapy of metastatic osteogenic sarcoma. Cancer 30:1627–1631, 1972.
2. JAFFE N, FARBER S, TRAGGIS D, ET AL. Favorable response of osteogenic sarcoma to high dose methotrexate with citrovorum rescue and radiation therapy. Cancer 31:1367–1373, 1973.
3. JAFFE N, FREI E (III), TRAGGIS D, ET AL. Adjuvant methotrexate-citrovorum factor treatment of osteogenic sarcoma. N Engl J Med 291:994–997, 1974.
4. JAFFE N, FREI E (III), TRAGGIS D, ET AL. Weekly high-dose methotrexate-citrovorum factor in osteogenic sarcoma: presurgical treatment of primary tumor and of overt pulmonary metastases. Cancer 39:45–50, 1977.
5. JAFFE N, LINK M, WATTS H, ET AL. The role of high-dose methotrexate in osteogenic sarcoma. J Natl Cancer Inst. In press.
6. KAPLAN EI, and MEIER P. Nonparametric estimation from incomplete observations. J Am Statist Assoc 53:457, 1958.
7. JAFFE N, and TRAGGIS D. Toxicity of high-dose methotrexate (NSC-740) and citrovorum factor (NSC-3590) in osteogenic sarcoma. Cancer Chemother Rep 6:31–36, 1975.
8. ROSEN G, MURPHY ML, HUVOS AG, ET AL. Chemotherapy, en bloc resection, and prosthetic bone replacement in the treatment of osteogenic sarcoma. Cancer 37:1–11, 1976.
9. JAFFE N, WATTS H, FELLOWS KE, ET AL. Local en bloc resection for limb preservation. Cancer Treat Rep 62:217–223, 1978.
10. FREI E (III), JAFFE N, GERO M, ET AL. Adjuvant chemotherapy osteogenic sarcoma: progress and perspectives. J Natl Cancer Inst 60:3210, 1978.
11. ROSEN G, MARCOVE RC, CAPARROS B, ET AL. Primary osteogenic sarcoma: the rationale for preoperative chemotherapy and delayed surgery. Cancer 43:2163–2177, 1979.

Selected Applications of Methotrexate Alone and in Combination in Osteosarcoma[1]

Charles B. Pratt*

SUMMARY

Review of the reported use of high-dose methotrexate (HDMTX) with citrovorum factor rescue (CF) for treatment of primary and metastatic osteosarcoma indicates the rarity of responses in clinical situations other than treatment of the primary tumor or isolated pulmonary metastases with doses of 7.5 g/m² or greater delivered on a weekly basis for 4 to 6 weeks. Adjuvant chemotherapy using HDMTX-CF at triweekly intervals has led to 2-year disease-free survival rates that are inferior to those obtained with combinations of agents including HDMTX-CF Adriamycin (Adria), with or without vincristine, cyclophosphamide, phenylalanine mustard, bleomycin, and dactinomycin. We concluded that although HDMTX-CF has a role in the treatment of primary and metastatic osteosarcoma, its use in combination with other active agents offers greater potential advantage to affected patients.

[Cancer Treat Rep 65 (Suppl. 1):107–111, 1981]

Data from studies with experimental animal tumor systems suggested that the effectiveness of methotrexate (MTX) might be increased by the administration of higher dosages that required rescue with citrovorum factor (CF) (1). Jaffe in 1971 (2) applied the methods of Djerassi (3) for the treatment of lung cancer to the treatment of patients with metastatic osteosarcoma. The results of these studies led to the prompt acceptance of the effectiveness of high-dose methotrexate with citrovorum factor rescue (HDMTX-CF) for the treatment of this tumor, which formerly had been considered to be resistant to the effects of antitumor agents. With the suggestion that the cellular efflux of MTX could be favorably enhanced by pretreatment of animal tumors with vincristine (VCR) (4), Jaffe added VCR to the regimen of HDMTX-CF. HDMTX-CF with VCR then was applied to the adjuvant treatment of patients with osteosarcoma following amputation of affected extremities (5). Similarly, and at the same time, Adriamycin (Adria) was introduced into adjuvant chemotherapy trials at the Roswell Park Memorial Institute and by members of the Cancer and Leukemia Group B (6), after determination of the beneficial effects of Adria on the treatment of patients with measurable indicators of disease activity.

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Results of treatment of osteosarcoma patients with HDMTX-CF have come from only scattered reports. The purpose of this report is to review the evidence of responses in patients with and without measurable indicators of disease activity who have received HDMTX-CF alone or in combination. These studies, which have been carried out at various institutions, will be individually cited within the tables of this paper and in accompanying references.

EFFECTS OF HDMTX-CF AS SINGLE AGENT CHEMOTHERAPY FOR OSTEOSARCOMA

Effects on Primary Osteosarcoma

In a paper by Jaffe (7) the results of treatment of primary osteosarcoma using HDMTX-CF weekly were described. An earlier paper, by Jaffe et al (8), detailed the effects on four patients treated with weekly HDMTX-CF in a dosage of 7,500 mg/m² for 4 to 6 weeks: three patients developed complete responses (CR) and one patient had evidence of partial response (PR).

Rosen et al (9), in their October 1979 report, detailed the treatment results achieved by 30 patients with primary or metastatic osteosarcoma following HDMTX-CF. In this report, 9 of 30 patients developed CR, 11 developed PR, and 10 had no evidence of response.

At St. Jude Children's Research Hospital four patients received between 1,250 and 15,000 mg/m² HDMTX-CF at weekly or biweekly intervals and had no response following these treatments of primary tumors of flat bones (10).

Effect of HDMTX-CF on Pulmonary Metastases

Using weekly HDMTX-CF in a dosage of 7,500 mg/m², Jaffe has previously reported effects on four patients, of whom two developed CR and one developed PR (8). Using triweekly HDMTX-CF for 14 patients, 2 developed CR and 3 had PR (8).

At St. Jude Children's Research Hospital five patients received 2,500 to 15,000 mg/m² of HDMTX-CF at weekly or biweekly intervals; one patient developed a CR of 3 weeks' duration, two developed PR (including one patient who had a decrease in pleural effusion), and two patients failed to respond (10).

Ambinder et al reported stabilization of disease activity, for 23 months, in one of seven patients treated for metastatic osteosarcoma (11).

Effect of HDMTX-CF on Primary Osteosarcoma and Associated Pulmonary Metastases

The effect of weekly HDMTX-CF on the primary tumor as well as pulmonary metastases was evaluated in two patients at our hospital. Both patients developed PR (10).

Effect of HDMTX-CF in Increased Dosages and Frequency for Pulmonary or Pulmonary-Bony Metastases After Previous HDMTX Treatments

Jaffe observed PR of pulmonary lesions in two of five patients treated with weekly HDMTX-CF following previous treatment that included triweekly HDMTX-CF (8).

At our hospital four patients with pulmonary metastases and two with pulmonary and bony metastases following adjuvant combination chemotherapy including HDMTX-CF failed to develop responses with HDMTX-CF infusions administered in increased dosages at weekly or biweekly intervals (10).

Effect of HDMTX-CF Following Resection of Pulmonary Metastases After Failure of Adjuvant Combination Chemotherapy Including HDMTX-CF

Also at our hospital, six patients had unilateral thoracotomies for resection of pulmonary lesions that developed during adjuvant combination chemotherapy including HDMTX-CF (10). Each of five patients with unilateral disease developed other pulmonary metastases between 1 and 10 months, while receiving HDMTX-CF. One patient who had five lesions removed from one lung remains free of disease 51+ months following resection and secondary treatment with HDMTX-CF.

Effect of HDMTX-CF in Adjuvant Chemotherapy

Jaffe applied the information and results of treatment of measurable metastatic osteosarcoma to the adjuvant treatment of 12 patients, who received HDMTX-CF with VCR (12) (table 1). Forty-two percent of the 12 patients remained disease free for more than 2 years. There were, however, patients who developed pulmonary metastases as late as 4 years from diagnosis.

TABLE 1.—Adjuvant chemotherapy for osteosarcoma using HDMTX alone

Treatment	Number patients	Percentage overall	2-year survival disease-free	References
HDMTX-VCR	12		42	12
HDMTX-VCR±BCG	39	76	38	13

HDMTX = High-dose methotrexate-citrovorum factor
VCR = Vincristine
BCG = Bacillus Calmette Guerin

Rosenberg and his associates at the National Cancer Institute were not impressed with the disease-free survival rate of 38%, which their patients obtained with the use of HDMTX-CF with VCR, and with or without immunotherapy with Bacillus Calmette Guerin (BCG) (13) (table 1). The overall 70% 2-year survival probably reflects the aggressive use of thoracotomies for resection of pulmonary metastases (14).

EFFECTS OF HDMTX-CF AS USED IN COMBINATION CHEMOTHERAPY OF OSTEOSARCOMA

This section will consider results of the treatment of primary osteosarcoma, the pulmonary metastases of osteosarcoma, the primary and pulmonary metastases of osteosarcoma, pulmonary and bony metastases of osteosarcoma, and multifocal osteosarcoma with combinations of agents including HDMTX-CF. Additionally, the results of adjuvant chemotherapy using combinations of agents including HDMTX-CF will be reviewed.

Histologic Effect on Primary Osteosarcoma

Rosen has recently updated the data regarding the histologic effects of preoperative chemotherapy on the primary extremity tumors prior to bone replacement procedures of 31 patients receiving preoperative HDMTX-VCR-Adria (Protocol T-5) (15); 16 demonstrated minimal or no effects on tumor viability; 9 of these 16 patients are surviving, 6 without active dis-

ease; for 15 patients who had no evidence or only scattered evidence of histologically viable tumor within the resected specimen, all are disease-free survivors. For 20 patients receiving preoperative chemotherapy by the T-7 protocol, including bleomycin-cyclophosphamide-dactinomycin-HDMTX-CF-VCR-Adria, 16 patients had either no evidence or only scattered evidence of histologically viable tumor (14).

Effect on Pulmonary, Bony, and Soft Tissue Metastases

In 1974 Rosen reported the use of HDMTX-CF alternating with Adria for the treatment of patients with metastatic osteosarcoma, including *pulmonary metastases* with and without the primary tumor, *bony metastases,* and *soft tissue metastases* (16). One of five patients with pulmonary metastases only achieved a PR, whereas two additional patients had stabilization of disease. Two of four patients with lung plus bony metastases developed PR. One additional patient with bone and soft tissue metastases had a PR.

In 1978 Rosen reported on the value of combination chemotherapy, including HDMTX-CF, VCR, Adria, and cyclophosphamide before and after thoracotomies for the pulmonary metastases of osteosarcoma (17). Thoracotomy with initial complete removal of metastases followed by postoperative "adjuvant" chemotherapy using these agents resulted in prolonged survival of 10 of 14 patients with a median of 33 months (range 24 to 56 months). Preoperative chemotherapy with these same agents followed by multiple thoracotomies and postoperative continuation of these agents has resulted in disease-free survival of 12 of 31 patients for 6 to 41+ months (median 19+ months).

At our hospital, treatment with HDMTX-CF-Adria-cyclophosphamide resulted in CR for one of five children with lung metastases only, PR in one patient with lung plus nodal metastases, stabilization of disease in one patient with lung and bony mestastases, stabilization of disease in one patient with multifocal osteosarcoma, and no response for one patient with primary tumor with nodal metastases (10).

Effect on Primary Unresectable Osteosarcoma

HDMTX-CF-Adria-cyclophosphamide in combination was given to two patients with unresectable primary osteosarcoma of flat bones; neither patient developed evidence of response with HDMTX-CF doses of 2,500 or 5,000 mg/m² (10). Two patients who had had incomplete resections of tumor responded for 10 and 40 months respectively.

HDMTX-CF in Combination Adjuvant Chemotherapy

Of 22 patients treated with HDMTX-CF-VCR with Adria at the Sidney Farber Cancer Center, 73% remained disease free at 2 years (table 2) (12).

TABLE 2.—Adjuvant chemotherapy for osteosarcoma using HDMTX in combination with other agents

Treatment	Number patients	Percentage overall	2-year survival disease-free	References
HDMTX-VCR-Adria	22		73	12
HDMTX-VCR-Adria-Cyclo				
Amputation (T2)	23	72		15
En bloc resection (T5)	31	85		15
En bloc resection + Bleo-Dact (T7)	61		*88	15
HDMTX-VCR-Adria-Cyclo-PAM				
COMPADRI II	60		51	18
COMPADRI III	44		42	18
HDMTX-VCR-Adria	18	35	26	19
HDMTX-VCR-Adria ± BCG	17	83	**60	20
HDMTX-VCR-Adria-Cyclo	29	70	50	21
HDMTX-Adria-Cyclo	26	65	55	22
HDMTX-Adria	31		†50	23

HDMTX = High-dose methotrexate-citrovorum factor
VCR = Vincristine
Adria = Adriamycin
Cyclo = Cyclophosphamide
Bleo = Bleomycin
Dact = Dactinomycin
PAM = Phenylalanine mustard

* = At 22 months
** = At 18 months
† = At 12 months

Rosen has projected an impressive 88% disease-free survival for patients treated pre- and postamputation with multiple agent chemotherapy (T-7 protocol) (15). Two-year disease-free survival for other adjuvant chemotherapy trials has ranged from 26% to 60% (18–23). However, the median 2-year disease-free survival from these studies is 51%, which is better than the 36% 2-year metastasis-free survival results achieved with surgery alone for 37 patients treated in uncontrolled trials at the Mayo Clinic between 1969 and 1974 (24).

It is known that many other investigators are using HDMTX-CF in combination adjuvant chemotherapy protocols in the United States, England, Germany, France, Spain, Austria, Japan, and Australia. It was not the intention to omit reference to these important studies, yet the long-term results with followup of patients have yet to be published for comparison with previous studies.

DISCUSSION

With the indication of favorable responses of pulmonary metastases in patients with osteosarcoma following treatment with HDMTX-CF (2), there was acceptance of this method for the treatment of this tumor. There were indications that patients who failed to respond at MTX dosages of 2,500 mg/m² often developed responses when the dosages were increased to 5,000 or 6,000 mg/m². Yet there were no

formal studies of dose response relationships nor was there an establishment of minimal effective or maximally tolerated dosages of the agent, the usual criteria applied to phase I and II evaluations of new anticancer agents. Additionally, there were no larger classical phase III clinical studies by single institutions or groups of institutions to determine the merits of this agent in concurrent trials using low, moderate, or high doses of MTX with or without CF.

In adjuvant chemotherapy trials using HDMTX-CF with VCR, with or without BCG, the 2-year disease-free survival rates of 42% and 38% as reported by Jaffe (12) and Rosenberg (13) respectively are less than the reported disease-free survival rates for patients treated additionally with other active agents in institutions except at Mayo Clinic (19) and by the COMPADRI III regimen of the Southwest Oncology Group (18). The results of adjuvant chemotherapy for the 411 patients treated at the various institutions, as summarized in tables 1 and 2, vary remarkably, with overall survival ranging from 35% to 85%, and disease-free survival ranging from 26% to 88% (6,9,12,13,15,18–23). The increase in survival rate of osteosarcoma patients treated at the Mayo Clinic between 1969 and 1974 (24) was not in agreement with survival of patients treated at other institutions during the same period (25). Obviously the success of adjuvant chemotherapy must be measured in terms of disease-free survival percentages rather than overall survival percentages because increased numbers of patients are surviving following resection of pulmonary metastases, which may develop many months after the completion of adjuvant chemotherapy (26). Late metastases have developed in our patients up to 32 months from diagnosis, in Rosen's patients as late as 33 months (15), in Sutow's patients as late as 36 months (18), and in Jaffe's patients as late as 48 months (12).

Djerassi has observed that the antitumor effect of HDMTX is not dependent upon the development of MTX-related toxicity (27). The same is probably true of other agents that are currently being used in the adjuvant chemotherapy of osteosarcoma, including Adria, cyclophosphamide, and cis-platinum diamminedichloride (CDDP). One must therefore question the need for extremely high and potentially toxic doses of MTX, or other agents, in the treatment of osteosarcoma in the adjuvant situation, in comparison to the treatment of patients with measurable indicators of disease activity.

With pediatric as well as adult neoplasms, that occur with greater frequency than osteosarcoma, great emphasis has been placed on specially designed treatment programs for individuals who may be at high risk for lack of response or for early and predictable recurrence of disease. Approximately one-third of osteosarcoma patients admitted to large treatment centers do not qualify for admission to adjuvant chemotherapy protocol schemes, because of evidences of metastatic tumor or other extenuating circumstances such as unresectable primary tumor. It is from these patients that the results of primary treatments should be carefully assessed for application into future adjuvant chemotherapy programs.

CONCLUSION

Review of the reported use of HDMTX-CF for the treatment of primary and metastatic osteosarcoma indicates the relative rarity of responses in clinical situations other than the treatment of the primary tumor before resection or amputation, or in the treatment of isolated pulmonary metastases, using dosages of 7,500 mg/m^2 or greater delivered on a weekly basis for 4 to 6 weeks (2,8–11). Adjuvant chemotherapy using HDMTX-CF at triweekly intervals has led to 2-year disease-free survival rates that are inferior to those obtained using combinations of agents, including HDMTX-CF and Adria, with or without VCR, cyclophosphamide, phenylalanine mustard, bleomycin, and dactinomycin. We concluded that although HDMTX-CF has a role in the treatment of primary and metastatic osteosarcoma, and in the adjuvant situation, its use in combination with other active agents offers greater potential advantage to affected patients.

REFERENCES

1. Goldin A, Mantel N, Greenhouse SW, et al. Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of amenopterin in mice. Cancer Res 14:43–48, 1954.
2. Jaffe N. Recent advances in the chemotherapy of metastatic osteogenic sarcoma. Cancer 30:1627–1631, 1972.
3. Djerassi I, Rominger CJ, Kim JS, et al. Phase I study of high doses of methotrexate with citrovorum factor in patients with lung cancer. Cancer 30:22–30, 1972.
4. Zager RF, Frisby SA, and Oliverio VT. The effects of antibiotics and cancer chemotherapeutic agents on cellular transport and antitumor activity of methotrexate in L1210 murine leukemia. Cancer Res 33:1670–1676, 1973.
5. Jaffe N, Frei E (III), Traggis D, et al. Adjuvant methotrexate-citrovorum factor treatment of osteogenic sarcoma. N Engl J Med 291:994–997, 1974.
6. Cortes EP, Holland JF, Wang JJ, et al. Amputation and adriamycin in primary osteosarcoma. N Engl J Med 291:998–1000, 1974.
7. Jaffe N. High-dose methotrexate in Osteogenic Sarcoma Adjuvant Chemotherapy and Limb Salvage Results. Cancer Treat Rep 65 (Suppl. 1):99–106, 1981.
8. Jaffe N, Frei E (III), Traggis D, et al. Weekly high-dose methotrexate-citrovorum factor in osteogenic sarcoma. Cancer 39:45–50, 1977.
9. Rosen G, Juergens H, Nirenberg A, et al. Response of primary osteogenic sarcoma to high-dose methotrexate-single agent chemotherapy. *In* Proceedings of the 11th International Congress of Chemotherapy and 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, MA, October 1–5, 1979, (Abstr) 535.
10. Pratt CB, Howarth C, Ransom JR, et al. High-dose methotrexate alone and in combination therapy for measurable primary or metastatic osteosarcoma. Cancer Treat Rep 64:11–20, 1980.
11. Ambinder EP, Perloff M. Ohnuma T, et al. High dose methotrexate followed by citrovorum factor reversal in patients with advanced cancer. Cancer 43:1177–1182, 1979.
12. Jaffe N, Frei E (III), Watts H, et al. High-dose methotrexate in osteogenic sarcoma: a 5-year experience. Cancer Treat Rep 62:259–264, 1978.

13. Rosenberg SA, Chabner BA, Young RC, et al. Treatment of osteogenic sarcoma. I. Effect of adjuvant high-dose methotrexate after amputation. Cancer Treat Rep 63:739–751, 1979.
14. Rosenberg SA, Flye MW, Conkle D, et al. Treatment of osteogenic sarcoma. II. Aggressive resection of pulmonary metastases. Cancer Treat Rep 63:753–756, 1979.
15. Rosen G, Marcove RC, Caparros B, et al. A primary osteogenic sarcoma. The rationale for preoperative chemotherapy and delayed surgery. Cancer 43:2163–2177, 1979.
16. Rosen G, Suwanisirikul S, Kwon C, et al. High dose methotrexate with citrovorum factor rescue and Adriamycin in childhood osteogenic sarcoma. Cancer 33:1151–1163, 1974.
17. Rosen G, Huvos AG, Mosende C, et al. Chemotherapy and thoracotomy for metastatic osteogenic sarcoma: a model for adjuvant chemotherapy and the rationale for the timing of thoracic surgery. Cancer 41:841–849, 1978.
18. Sutow WW, Gehan EA, Dyment PG, et al. Multidrug adjuvant chemotherapy for osteosarcoma: interim report of the Southwest Oncology Group Studies. Cancer Treat Rep 62:265–270, 1978.
19. Gilchrist GG, Ivins JC, Ritts RE (jr), et al. Adjuvant therapy for non-metastatic osteogenic sarcoma: an evaluation of transfer factor versus adjuvant chemotherapy. Cancer Treat Rep 62:289–294, 1978.
20. Eilber FR, Grant T, and Morton DL. Adjuvant therapy for osteosarcoma: preoperative and postoperative treatment. Cancer Treat Rep 62:213–216, 1978.
21. Etcubanas E, and Wilbur JR. Adjuvant chemotherapy for osteogenic sarcoma. Cancer Treat Rep 62:283–287, 1978.
22. Pratt CB, Howarth C, Ransom JL, et al. High dose methotrexate in combination chemotherapy for childhood tumors. *In* Proceedings of the High Dose Methotrexate International Workshop, June 13–14, 1978, Firenze, Italy, (Periti P, ed). Firenze, Editrice Guintina, 1978, pp 233–242.
23. Cortes EP, Necheles TF, Holland JF, et al. Adriamycin (ADM) alone versus ADM and high dose methotrexate citrovorum factor rescue (HDMTX-CF) as adjuvant to operable primary osteosarcoma (OS): a randomized study by cancer and leukemia group B (CALGB). Proc Am Assoc Cancer Res and ASCO 24:412, 1979.
24. Taylor WF, Ivins JC, Dahlin DC, et al. Trends and variability in survival from osteosarcoma. Mayo Clinic Proc 53:695–700, 1978.
25. Frei E, and Polli E. Discussion. *In* Proceedings of the High Dose Methotrexate International Workshop, June 13–14, 1978, Firenze, Italy, (Periti P, ed). Firenze, Editrice Guintina, 1978, pp 229–232.
26. Muggia F, Catane R, Lee YJ, et al. Factors responsible for therapeutic success in osteosarcoma: a critical analysis of adjuvant trial results. *In* Adjuvant Chemotherapy of Cancer II (Salmon S, and Jones S, eds). New York, NY, Grune & Stratton, 1979, pp 383–390.
27. Djerassi I. Discussion. *In* Proceedings of the High Dose Methotrexate International Workshop, June 13–14, 1978, Firenze, Italy, (Periti P, ed). Firenze, Editrice Guintina, 1978, pp 173–174.

U.K. and European Trials of Chemotherapy in Osteosarcoma

J. A. Bullimore*

SUMMARY

Three current studies of treatment of osteosarcoma are reviewed and the results of a small completed study of adjuvant chemotherapy are given. The Medical Research Council has 152 patients in a continuing trial comparing two differing adjuvant chemotherapy schedules. The European Organisation of Research on the Treatment of Cancer, together with the International Society of Paediatric Oncology, are undertaking a three-arm trial of chemotherapy, lung irradiation, and chemotherapy plus lung irradiation, as adjuvant treatments in osteosarcoma, in which 85 patients have been entered as of September 1979. The Bristol Bone Tumour Registry treated 11 patients with a regimen consisting of intermediate-dose methotrexate and doxorubicin given for 1 year postoperatively, and have 64% of patients alive and disease free at a followup period of 42 to 62 months. A further study, based in Bristol, of chemotherapy and surgical excision of lung metastases has 8 of 14 patients free of disease, 3 to 35 months from thoracotomy.

[Cancer Treat Rep 65 (Suppl. 1):113-117, 1981]

Two large studies of adjuvant treatment in osteosarcoma are currently under way in Europe. The Medical Research Council (MRC) is running a randomized study in Britain, comparing two differing adjuvant chemotherapy schedules. The European Organisation for Research on the Treatment of Cancer (EORTC) has started a study in which France, Holland, Germany, Belgium, and Spain are collaborating, and is also comparing differing adjuvant treatments. Prior to the start of the MRC trial, the Bristol Bone Tumour Registry (BTR) studied the effects of an adjuvant chemotherapy regimen on 11 patients with osteosarcoma, and compared the results with a historical series. In addition, there is a small study continuing at present, based in Bristol, attempting to assess the efficacy of chemotherapy and surgical removal of pulmonary lesions in patients with metastatic disease.

PATIENTS AND METHODS

MRC and EORTC Trials

The MRC trial was started in January 1975 and, up to September 1979, 152 patients have been entered; patients eligible must be under 40 years of age, having an osteosarcoma with no overt metastatic spread. The primary tumor may be treated by radical surgery or by the Cade method of radical radiotherapy followed by surgical excision (the former method is preferred). Adjuvant chemotherapy is started within 2 weeks of primary surgery, or with radiotherapy if the Cade method is used. There are two arms of the trial, each consisting of a differing chemotherapy schedule. In one arm vincristine (VCR) 1.5 mg/m² (maximum dose 2 mg) is given iv half an hour before a bolus injection of methotrexate (MTX) 200 mg/m². Citrovorum factor rescue (CF) is started 24 hours later using calcium leucovorin (LV) 15 mg tablets, every 6 hours for six doses. The drugs are repeated every 3 weeks for a total of 18 times; that is, over a period of approximately 54 weeks. In the second arm VCR–MTX–CF are given as in the first arm, but once every 6 weeks, with doxorubicin[1] 60 mg/m² iv given 3 weeks after each VCR–MTX–CF administration. The overall treatment time is the same as in the first arm, that is, 54 weeks. At the trial's inception, a great deal of discussion had taken place regarding whether or not a "no adjuvant therapy" control arm should be included, as was initially the intention. Publication of papers by Jaffe et al (1) and Cortes et al (2) in 1974 and 1975 claiming improved early survival figures using adjuvant chemotherapy made many clinicians feel that it would be unethical to have a "no treatment" arm. However, the doubts raised by the publication of the Mayo Clinic's (3)

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[1] Doxorubicin: trade name Adriamycin.

results for 1963 to 1974 have caused us to wonder if we should have had the courage to carry out our original intention.

The current EORTC trial of adjuvant chemotherapy for osteosarcoma commenced in January 1978, and as of September 1979, 85 patients have been entered. The EORTC has now joined forces with the International Society of Paediatric Oncology (SIOP) and it is hoped that an accession rate of 300 patients will be achieved by 1983. The rationale for this trial is as follows: A study of lung irradiation as an adjuvant therapy was carried out by the EORTC (4), and the results published in 1978 indicated that at 5 years 43% of the patients receiving lung irradiation remained disease free as opposed to 28% in the control group who received no lung irradiation. It is felt that, as lung irradiation appears to improve survival rates, if it is given in addition to adjuvant chemotherapy, then improved survival rates may be achieved with less morbidity and a shortening of overall treatment time. Lung irradiation given after completion of chemotherapy cannot cause recall radiation pneumonitis with its increased risk of lung damage. The risk of cardiotoxicity is also reduced if doxorubicin and radiation to the heart muscle are not given concurrently.

To be eligible for protocol entry, patients must be under 30 years of age with an osteosarcoma of a limb with no clinically detectable metastases. Patients with lung metastases will be studied separately. The primary tumor is treated by ablative surgery. There are three arms to the trial and patients are randomly allocated to these; there is no group receiving no adjuvant therapy, ie, no control group. Adjuvant therapy must be started within 4 weeks of surgery.

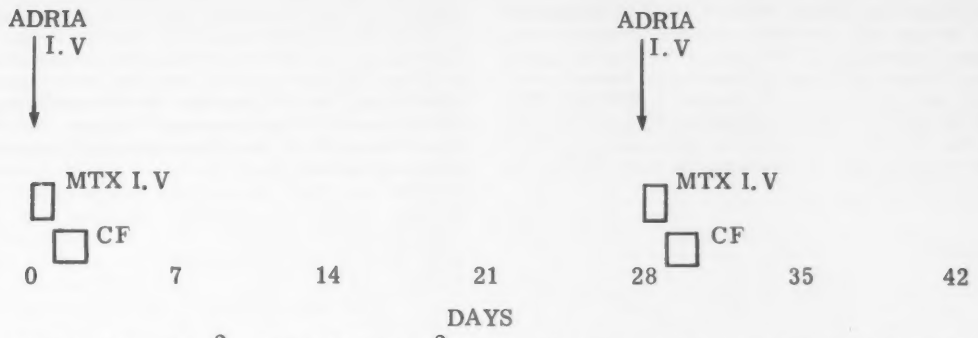
Patients in the first group receive chemotherapy alone for approximately 41 weeks, the second group receives radiation to both lungs over 12 days, and the third group receives chemotherapy over 9 weeks, followed by lung irradiation. The lung irradiation in the second and third groups consists of 2,000 rad midplane dose, given to the whole of both lungs in 10 fractions over 12 days using megavoltage equipment. The chemotherapy used in the third group is the same as that used during the first 9 weeks of adjuvant treatment in the first group, that is, VCR 1.5 mg/m^2 (maximum 2 mg) followed by MTX 6 g/m^2 as an infusion over 6 hours, and CF, alternating every 2 weeks with doxorubicin 70 mg/m^2 iv to week 9 so that a total of three VCR-MTX-CF administrations and two doxorubicin injections are given. In the first group chemotherapy is continued after the ninth week, cytotoxic drugs being given every 2 weeks; in week 11 cyclophosphamide 1.2 g/m^2 iv is given; in week 13 doxorubicin; in week 15 cyclophosphamide; in week 17 VCR-MTX-CF. This 8-week cycle of drugs is repeated four times.

BTR Adjuvant Chemotherapy Trial

The BTR, encouraged by reports of high-dose methotrexate (HDMTX) and doxorubicin obtaining resolution of lung metastases in osteosarcoma, started a study in January 1974 of adjuvant chemotherapy. Eligible patients were under 30 years of age with an osteosarcoma of long bones with no demonstrable metastases. Primary tumors were treated by ablative surgery, and chemotherapy was started within 2 weeks of operation. A consecutive series of patients with similar tumors was taken from the BTR records and used as a historical control group. Chemotherapy consisted of doxorubicin 60 mg/m^2 iv followed by a 24-hour infusion of MTX 200 mg/m^2 with CF. These drugs were repeated every 4 weeks for 12 cycles (fig 1). The study ran from January 1974 to November 1976, at which time it was closed and subsequent patients were entered into the MRC trial. Eleven patients were treated, and in February 1979, seven of these were alive and disease free, that is, 64% at a minimum followup of 42 months and a maximum of 62 months. The comparable figure for the control group is 20%. One of the patients in the study group had a solitary lung metastasis removed in November 1976, and received further chemotherapy (fig 2). The numbers in this study group are too small to carry much weight, but a few points are of interest: it is the only study in which intermediate dose MTX and doxorubicin have been given within the same 24 hours; no serious toxicity was encountered, yet the results compare favorably with studies using much higher doses of drugs with the accompanying increased morbidity and hazards. One patient has had two normal children after completing therapy, the first being born 1 year from the end of treatment.

Bristol Trial for Lung Metastases

In this study the value of chemotherapy and surgical excision of metastases is being assessed. The protocol consists of: phase I, in which MTX is given weekly for 4 weeks starting at a dose of 1 g/m^2 given as a 6-hour infusion with CF rescue, and increasing to 4 g/m^2 by the fourth week. VCR 1.5 mg/m^2 (2 mg maximum) is given 30 minutes before the start of each MTX infusion. Surgical excision of lung metastases is carried out approximately 10 days from the fourth MTX infusion. Phase II of chemotherapy is started approximately 10 days after thoracotomy. If



ADRIA 60 mg/m^2 MTX 200 mg/m^2 over 24 hours. CF 10-15 mg 6 hourly x 6

ADRIA = ADRIAMYCIN MTX = METHOTREXATE CF = CITROVORUM FACTOR

BTR ADJUVANT CHEMOTHERAPY PROTOCOL FOR OSTEOSARCOMA

FIGURE 1.—BTR adjuvant chemotherapy regimen for osteosarcoma.

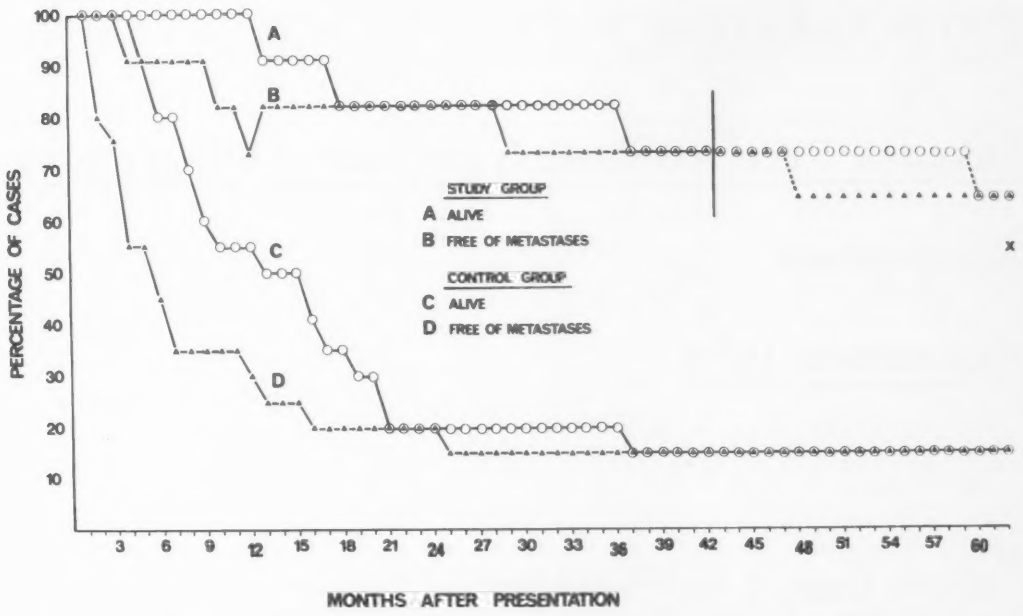
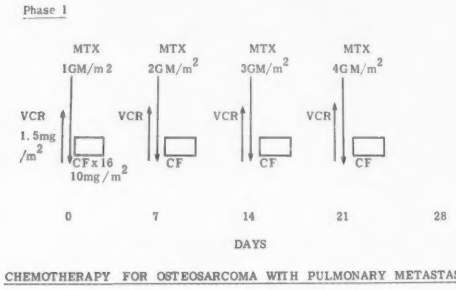


FIGURE 2.—Results of BTR adjuvant chemotherapy study. NB: The dip in B at 12 months is due to the patient who had a solitary lung metastasis removed and has since remained disease free.

the patient has already received maximum doxorubicin dosage with previous adjuvant chemotherapy, phase II consists of VCR and MTX 4 g/m² given every 3 weeks for 1 year. If doxorubicin has not previously been given, phase II consists of VCR and MTX 4 g/m² on Day 1 and doxorubicin 50 mg/m² on Day 8; this schedule is repeated on a 4-week cycle for 1 year (figs 3a and 3b). Between November 1976 and September 1979, 14 patients have been treated, and 8 of these are surviving apparently free of disease. The followup period from last thoracotomy ranges from 3 to 35 months; four patients have had more than one thoracotomy, two of these were for bilateral lung deposits present when metastatic disease was first noted; one patient died from septicemia associated with drug toxicity (fig 4).



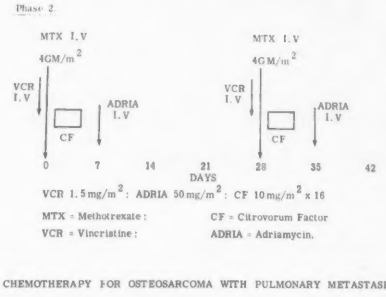


FIGURE 3a and 3b.—Bristol chemotherapy regimen used with resection of pulmonary metastases.

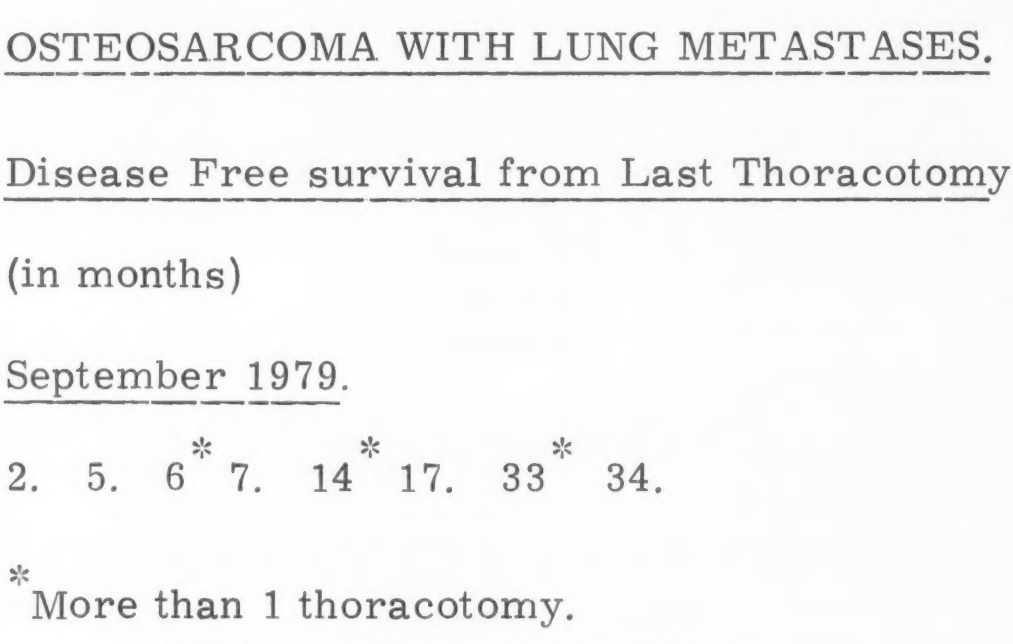

OSTEOSARCOMA WITH LUNG METASTASES.
Disease Free survival from Last Thoracotomy
(in months)
September 1979.
2. 5. 6* 7. 14* 17. 33* 34.
*More than 1 thoracotomy.

FIGURE 4.—Time of survival since thoracotomy of disease-free patients (8 of 14 patients treated).

CONCLUSION

In order to provide answers that are statistically significant and will give scientifically acceptable proof of efficacy or nonefficacy of a new form of treatment in a rare disease such as osteosarcoma, multicenter studies such as the MRC and EORTC trials are essential. Nevertheless, smaller studies such as those undertaken in Bristol, which have historical controls, may provide evidence of benefit from new protocols that may, in turn, form the basis of larger and more scientifically acceptable trials.

REFERENCES

1. Jaffe N, Frei E (III), Traggis D, et al. Adjuvant methotrexate and citrovorum factor treatment of osteogenic sarcoma. N Engl J Med 291:994–997, 1974.
2. Cortes EP, Holland JF, Wang JJ, et al. Adriamycin (NSC-123127) in 87 patients with osteosarcoma. Cancer Chemother Rep (Part 3) 6:305–311, 1975.
3. Taylor WF, Ivins JC, Dahlin DC, et al. Osteogenic sarcoma experience at the Mayo Clinic, 1963–1974. *In* Immunotherapy of Cancer: Present Status of Trials in Man, New York, NY, Raven Press, 1978, pp 257–269.
4. Breur K, Cohen P, Schweisguth O, et al. Irradiation of the lungs as an adjuvant therapy in the treatment of osteosarcoma of the limbs. Results of a controlled clinical trial by the clinical cooperative group on radiotherapy of the EORTC. Eur J Cancer 14:461–467, 1978.

Combination Chemotherapy of Childhood Non-Hodgkin's Lymphomas

M. G. Mott*

SUMMARY

The rationale for the treatment of the non-Hodgkin's lymphomas in childhood is discussed. Results from recent trials of combination chemotherapy are given, and the treatment strategy of the United Kingdom Children's Cancer Study Group is described.

[Cancer Treat Rep 65 (Suppl. 1):119–123, 1981]

The prognosis for non-Hodgkin's lymphomas (NHL's) of childhood has improved markedly since the introduction of intensive combination chemotherapy as primary treatment. The substantial cure rates in Hodgkin's disease by radiotherapy alone in carefully staged patients fostered the hope that this might also be achieved in NHL. This proved not to be the case, at least in childhood, because of the difference in the natural history of these disorders.

Hodgkin's disease is a relatively indolent process and spread occurs by involvement of contiguous lymph nodes, but these features are not shared by NHL. The NHL's that occur in childhood also differ considerably from those of adults both in morphology and in their clinical behavior. More than 95% of childhood tumors are diffuse, so nodular histology is a rarity. Furthermore, the disease is systemic at the time of diagnosis in the great majority of cases. This is the major reason that radiation alone cures only about half of the 20% of patients who present with strictly localized disease (1).

NHL is clearly more than one disease. The recent explosion of knowledge of the structure and function of the lymphoid system has helped considerably to develop a better understanding of the lymphomas and of their origins. Cytomorphological classifications have been clarified and amplified by new techniques that have aided the differentiation of malignant cell lines that originate from T and B lymphocytes and monocytes, and these can be seen to correlate well with characteristic clinical syndromes.

RATIONALE OF PRESENT TREATMENT

The modern management of NHL's of childhood is based on the known natural history of the disease.

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Generalized Disease

At least 80% of patients have generalized disease at diagnosis. Curative treatment, therefore, requires intensive combination chemotherapy. Chemotherapy with single agents or standard adult combinations such as COP has been relatively ineffective in childhood NHL's and the use of standard antileukemic regimens has also proved disappointing (2–5). The 2-year survival remained poor in all reported series until the recent advent of intensive combination chemotherapy. The drug combinations used are designed to provide an additive or synergistic effect against the tumor, while spreading toxicity to normal tissues through various organ systems, thus improving the therapeutic ratio. Most of the effective single agents against leukemia and lymphoma are combined in doses and schedules that are given to maximum tolerance at approximately 3-week intervals, allowing interim recovery from dose-limiting toxicities such as profound myelosuppression and immunosuppression.

The primary role of surgery is usually confined to providing adequate tissue for a definitive histological diagnosis, but may be extended to include removal of bulk tumor, for example, in abdominal primaries. Chemotherapy obviates the necessity for surgical staging procedures that are designed to identify microscopic disease outside of any planned radiation portal. The Ann Arbor staging system is of limited value because of the noncontiguous nature of dissemination and the high frequency of extranodal disease, especially involvement of bone marrow and meninges. Surgical staging is therefore irrelevant to the treatment of the great majority of patients and may cause a dangerous delay in the initiation of appropriate chemotherapy. It is possible that radiation may play an adjuvant role in the treatment of bulk disease where this is not managed more appropriately by surgery. In those patients with disseminated disease who receive radiotherapy as an adjuvant to che-

motherapy, the dose and field of radiation should not be such as to limit systemic chemotherapy, but should aim at helping to prevent local recurrence.

Localized Disease

About half of the 20% of patients with truly localized disease can be cured by radiation (1). Since chemotherapy appears capable of curing many patients with disseminated disease, it is uncertain whether those with localized disease, ie, stages I and II, should receive chemotherapy in addition to radiation from the outset, or if this should be introduced only if they later show evidence of relapse.

Central Nervous System Disease

About one-third of patients develop central nervous system (CNS) disease. The meninges are frequently the primary site of relapse in patients with stage I or II disease, particularly those who have had complete resection of a gastrointestinal primary. It is at present recommended that all patients with more widespread disease should receive some form of prophylactic treatment to the CNS, though it appears less effective in NHL than in leukemia in preventing overt CNS involvement (6,7).

COMBINATION CHEMOTHERAPY RESULTS

The first indication that intensive combination chemotherapy had substantially altered the previously poor prognosis for childhood NHL came from Memorial Hospital in New York (6,8–11). In 1971 the hospital modified its L2 leukemia protocol and applied it to lymphoma patients. Treatment commenced with an initial high dose of cyclophosphamide and simultaneous radiation to bulk tumor. All patients then continued on an intensive induction, consolidation, and maintenance chemotherapy program, which included intrathecal chemotherapy for CNS prophylaxis. Radiation was individualized, so that patients received doses ranging from 200 to 6,200 rad, though many received no radiation at all.

By 1976 Wollner was able to report that 42 of 58 patients (72%) were alive, 41 (70%) with no evidence of disease; 27 of the 42 survivors were off treatment, with a median survival of 45+ months (6). Sixteen patients had died, 12 of recurrence and dissemination and 4 of viral infections.

A recent update of these figures recorded a disease-free actuarial survival of 95% at a median followup of 26 months among 61 patients considered to have been adequately treated (10).

Sidney Farber Cancer Institute

The Boston experience with childhood NHL has also been reported recently (12). In that study 64 patients under 18 years of age were treated from January 1971 to December 1976, all patients with more than 5% of blasts in the bone marrow being excluded. Chemotherapy was variable during the first part of this time, but a standard regimen (APO) was used from 1973 to 1976. This regimen consisted of induction with prednisone 40 mg/m²/day, vincristine 1.5 mg/m²/week iv for four doses, and Adriamycin 75 mg/m² on weeks 1 and 4 (except for patients with gastrointestinal primaries). Maintenance chemotherapy consisted of 5-day courses of prednisone 120 mg/m²/day, mercaptopurine 225 mg/m²/day, with vincristine 1.5 mg/m², and Adriamycin 30 mg/m² iv on day 1 to a cumulative Adriamycin dose of 450 mg/m². Thereafter, methotrexate (MTX) was substituted at a dose of 7.5 mg/m² daily for 5 days. The radiation therapy used is detailed in the report.

Actuarial 5-year survival for this series was 61%, with a disease-free survival of 54%. Survival for patients before APO was introduced was 50% compared to 78% after its introduction. All recurrences were within 19 months of diagnosis. Of nine patients with abdominal primaries treated since 1971 with a combination of surgery, radiation, and chemotherapy, seven were currently surviving.

The same group has since reported on 12 children with initial mediastinal involvement treated by this chemotherapy regimen with the addition of craniocervical radiation for CNS prophylaxis, and they record an actuarial survival of 86% with a median followup of 41 months (13).

St. Jude Children's Research Hospital

From March 1975 to November 1978, 69 patients entered a study designed to investigate the effectiveness of adjuvant radiation therapy to bulk tumor in patients with disseminated disease, and to assess the effectiveness of CNS prophylaxis with cranial radiation and intrathecal MTX (14). Induction chemotherapy consisted of prednisone 40 mg/m²/day for 1 month, vincristine 1.5 mg/m²/week iv for 6 weeks, and cyclophosphamide 1.2 g/m² every 3 weeks for three doses. Patients with disseminated disease (St. Jude stages III and IV) had, in addition, Adriamycin 45 mg/m² every 3 weeks for three doses, ie, the CHOP regimen. Patients with stage I and stage II disease had involved field radiation to approximately 3,000 rad, whereas patients with stage III and stage IV disease were randomized to receive either no radiation or the same dose to areas of bulk disease. All patients except those with stage I disease or stage II completely resected gastrointestinal primaries were also randomized to receive 2,400 rad cranial radiation plus intrathecal MTX, 12 mg/m²/week for five doses, or no CNS prophylaxis. Maintenance chemotherapy consisted of mercaptopurine, 75 mg/m²/day, and MTX, 30 mg/m²/week for 18 months.

Results of this study to date show a 90%, 2-year disease-free survival for stage I and stage II disease and a 35% disease-free survival for stage III and stage IV disease (14). The addition of radiation in the

latter group has so far shown no benefit. There was, however, no evidence that the inclusion of radiation to bulk disease sites compromised the chemotherapy program, which was relatively gentle compared to other modern regimens.

Children's Hospital at Stanford

From May 1975 to April 1976, 12 patients entered a pilot study designed to test the efficacy of intensive combination chemotherapy as the primary treatment for childhood NHL, with or without the addition of adjuvant low-dose radiation to areas of bulk disease (15). Of the 12 patients, 9 were surviving 34+ to 45+ months from diagnosis and 8 were in continuous complete remission (CR). One patient with a mediastinal primary had a bone marrow relapse when off treatment at 32 months, but was in remission at 45+ months. There had been three deaths: one patient who presented with involvement of multiple bones, one with a mediastinal primary and bone marrow involvement at diagnosis, and one who had previously treated disease and who entered the study after bone marrow relapse.

Of the six patients who presented with a mediastinal mass, five are surviving 41+ to 45+ months, with four in continuous CR (16). Two of these four had no radiation, and two received 300 and 700 rad respectively to a localized area as emergency treatment for relief of respiratory and superior vena cava obstruction. The two patients who had bone marrow relapse at 27 and 32 months had received 450 rad to the mediastinum and 1,500 rad to the mediastinum and right hemithorax respectively.

United Kingdom Children's Cancer Study Group

Based on the pilot experience at Stanford and that of other centers, the Stanford protocol was modified and has been used as the basis for a trial organized by the United Kingdom Children's Cancer Study Group (UKCCSG). The primary aims of this trial are to confirm that intensive combination chemotherapy is the current treatment of choice in childhood NHL and to investigate whether adjuvant low dose (1,500 rad) radiation therapy to areas of bulk disease contributes anything further to such treatment. The treatment schedule is outlined in tables 1 and 2 and figure 1. The major change in this protocol compared to the Stanford Pilot Study has been the inclusion of intermediate-dose MTX with leucovorin (LV) rescue as consolidation therapy, based on the experience of Dr. I. Djerassi with this combination in Philadelphia (17).

TABLE 1.—UKCCSG NHL protocol (stages II, III, and IV) induction regimen

Intensive combination chemotherapy, with or without radiation to areas of bulk disease	
CHOP	
cyclophosphamide	1 g/m²
Adriamycin	50 mg/m² Day 1
vincristine	1.5 mg/m²
prednisolone	100 mg/m²—Days 1 to 5
	given every 3 weeks for two cycles
Cyt/TG	
cytosine arabinoside	100 mg/m² iv or subcutaneously every 12 hours for eight doses
thioguanine	75 mg/m² po daily for four doses
	given every 3 weeks for two cycles
Intrathecal MTX	10 mg/m² (maximum 12 mg) on Day 1 of each course

TABLE 2.—UKCCSG NHL protocol (stages II, III, or IV) consolidation and maintenance regimen

Consolidation

1. MTX 500 mg/m² (one-third iv push, two-thirds iv drip over 6 hours).
2. 24 hours after beginning of the infusion, folinic acid 12 mg/m² im or iv followed by three doses 6 mg/m² (iv or oral) over the next 24 hours.
3. Intrathecal MTX 10 mg/m² at the start of iv MTX infusions 1, 2, and 3 (given every 2 weeks for three cycles).

Randomization

With or without 1,500 rad of radiotherapy to area of bulk disease concurrently with consolidation chemotherapy.

N.B. Special category for patients with Sternberg sarcoma.
MTX 15 mg/m² oral daily × 4 every 2 weeks × 3 instead of iv MTX.
Intrathecal MTX on Day 1 of each course.
Cranial radiation, 1,760 rad in eight fractions of 220 rad over 2 weeks.

Maintenance

1. Cyclophosphamide/CCNU
   Cyclophosphamide 750 mg/m²
   CCNU 75 mg/m² po
2. VM 26 100 mg/m² iv
   MTX 500 mg/m² iv with citrovorum factor rescue (as in consolidation).

N.B. For Sternberg sarcoma patients who have received cranial radiation, MTX will not be given iv but orally at a dose of 12.5 mg/m² daily for 4 days.

3. CHOP
   Cyclophosphamide 750 mg/m²
   Adriamycin 40 mg/m²
4. Cytosine arabinoside/thioguanine
   Cytosine arabinoside 150 mg/m² iv or sc
   Daily × 5 days
   Thioguanine 75 mg/m² po

Given at 3-week intervals. 12-week maintenance cycle for 2 years.

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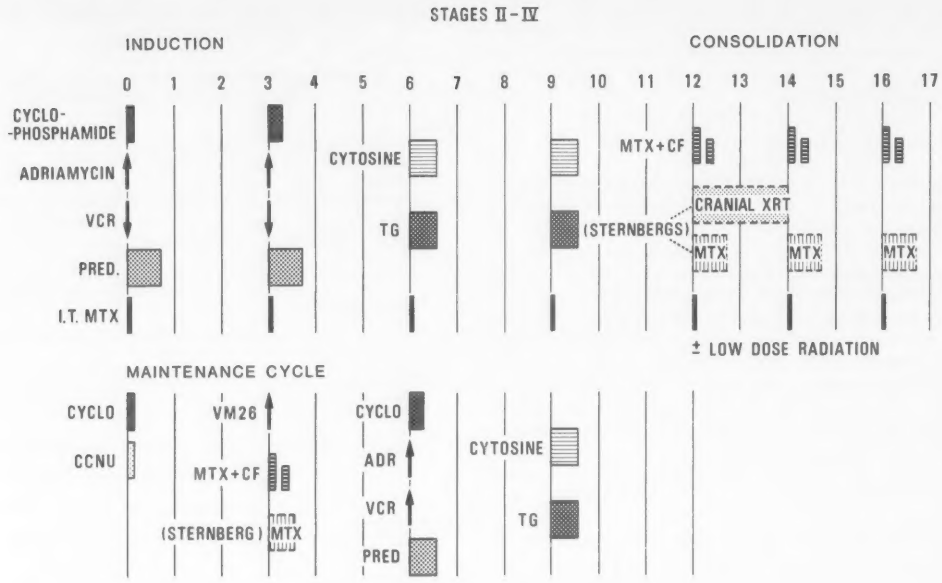

FIGURE 1.—UKCCSG NHL protocol.

Even at this intermediate dose, some patients are slow to excrete MTX, and maintain potentially toxic levels beyond the completion of LV rescue at 48 hours. Occasional mild evidence of toxicity and one fatal case of toxicity, in a pilot study patient who received the same dose and schedule of MTX and LV at weekly intervals, prompt the recommendation of routine assay of MTX levels at 48 hours, before LV rescue is discontinued.

The initial success of this program, particularly for T-cell lymphomas, had led to its further modification and intensification for use in patients with T-cell leukemia. While NHL is clearly different from acute lymphoblastic leukemia (ALL) (so it is no surprise that standard antileukemia treatment is relatively ineffective), T-cell lymphoma is almost certainly the same disease as T-cell leukemia, for which standard ALL treatment is also inadequate. Since intensive combination chemotherapy appears to be successful in the treatment of T-cell lymphoma, it seems logical to suggest that T-cell leukemia should be treated in a similar way.

From July 1977 to September 1979, 64 patients were entered on this study, and a further 22 patients on the more intensive modified pilot study for T-cell disease. It is too soon to evaluate the results, but preliminary analysis suggests that a similar improvement to that detailed in the above studies will be obtained.

CONCLUSION

The natural history of the childhood NHL's has changed in those given intensive combination chemotherapy as their primary treatment (table 3). We now must discover the incidence of recurrence in bulk tumor and of spread to bone marrow and CNS in the various disorders given such treatment, so that we can develop more selective combination chemotherapy programs for each disease, with or without adjuvant surgery and radiation, according to need.

TABLE 3.—Natural history of childhood NHL's

		Number of patients	Survival (percent)*	Disease-free survival (percent)*
MSKCC	1971–	58	72	70
SFCC	1971–1976	64	61	50 78
SJCRH	1975–1978	69		90 35
CHAS	1975–1976	12	75	66

*For details of duration, subgroups, etc, see text.
MSKCC—Memorial Sloan-Kettering Cancer Center.
SFCC—Sidney Farber Cancer Center.
SJCRH—St. Jude Children's Research Hospital.
CHAS—Children's Hospital at Stanford.

REFERENCES

1. Glatstein E, Kim H, Donaldson SS, et al. Non-Hodgkin's lymphomas. VI. Results of treatment in childhood. Cancer 34:204–211, 1974.
2. Luce JK, Gamble JF, Wilson HE, et al. Combined cyclophosphamide, vincristine and prednisone therapy of malignant lymphoma. Cancer 28: 206–217, 1971.
3. Bagley CM, DeVita VT, Berard CW, et al. Advanced lymphosarcoma; intensive cyclical combination chemotherapy with cyclophosphamide, vincristine and prednisone. Ann Int Med 76:227–234, 1972.
4. Pinkel D, Johnson W, and Aur RJA. Non-Hodgkin's lymphomas in children. Br J Cancer 31(Suppl II): 298–323, 1975.
5. Cebrian-Bonesana A, Schwartzman E, Roca-Garcia C, et al. Non-Hodgkin's lymphoma in children, an analysis of 122 cases from Argentina. Cancer 41:2372–2378, 1978.
6. Wollner N. Non-Hodgkin's lymphoma in children. Pediatr Clin N Am 23:371–378, 1976.
7. Sullivan MP. Treatment of lymphoma. Cancer 35:991–995, 1975.
8. Wollner N, Burchenal JH, Lieberman PH, et al. Non-Hodgkin's lymphoma in children; a comparative study of two modalities of therapy. Cancer 37:123–134, 1976.
9. Zea JM, Exelby PR, and Wollner N. Abdominal non-Hodgkin's lymphoma in childhood. J Pediatr Surg 11:363–369, 1976.
10. Wollner N, and Jereb B. Multidisciplinary LSA_2-L_2 protocol treatment of non-Hodgkin's lymphoma in children; a review of 73 cases. (Abstr) C-23, Proc Am Soc Clin Oncol, 1979.
11. Meadows AT, Jenkin RDT, Chilcote R, et al. Remission induction in childhood non-Hodgkin's lymphoma with cyclophosphamide, vincristine, prednisone, and intravenous methotrexate. (Abstr) C-234, Proc Am Soc Clin Oncol 19:365, 1979.
12. Carabell SC, Cassady JR, Weinstein JH, et al. The role of radiation therapy in the treatment of pediatric non-Hodgkin's lymphomas. Cancer 42:2193–2205, 1978.
13. Weinstein HJ, Vance ZB, Jaffe N, et al. Improved prognosis for patients with mediastinal lymphoblastic lymphoma. Blood 53:687–697, 1979.
14. Murphy SB, and Hustu HO. A randomised trial of combined modality therapy of childhood non-Hodgkin's lymphoma. Cancer 45:630–637, 1980.
15. Wilson JA, Mott MG, and Wilbur JR. Intensive combination chemotherapy of childhood non-Hodgkin's lymphoma. (Abstr)C-202, Proc Am Soc Clin Oncol 18, 1977.
16. Wilbur JR, Mott MG, and Wilson JA. Childhood non-Hodgkin's lymphoma of the mediastinum; combination chemotherapy as primary treatment. (Abstr)C-506, Proc Am Soc Clin Oncol 20:414, 1979.
17. Djerassi I, and Kim JS. Methotrexate and citrovorum factor rescue in the management of childhood non-Hodgkin's lymphomas. Cancer 38:1043–1051, 1976.

Methotrexate as a Single Agent and in Combination Chemotherapy for the Treatment of Non-Hodgkin's Lymphoma of Unfavorable Histology[1]

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SUMMARY

High-dose methotrexate (HDMTX) with citrovorum factor (CF) was used to treat non-Hodgkin's lymphomas either as a single agent in those patients who had previously failed to respond to conventional chemotherapy or in previously untreated patients in combination with bleomycin, Adriamycin, cytoxan, and vincristine. Twenty-five patients who had been previously treated and were refractory to cytotoxic chemotherapy were treated with HDMTX ranging between 1 and 7.5 g/m² as an iv infusion over 20 to 40 minutes. CF rescue was begun at 24 hours and was maintained orally every 6 hours at 10 mg/m² for a total of 72 hours. In instances where the 24-hour serum level of MTX was in excess of 1×10^{-7}M, the dose of CF was increased to 100 mg/m² every 3 hours until the serum MTX level fell into the appropriate range. Of the 25 patients, 13 (52%) responded. Response duration was brief, lasting a median of 2 months. Three patients had prolonged control lasting 13, 16, and 21 months respectively. Five of the six patients with central nervous system (CNS) involvement have demonstrated marked clearing of spinal fluid abnormalities and reversion of brain scans toward normal. Fifty-four previously untreated patients with diffuse histiocytic or undifferentiated lymphoma have been treated with combination chemotherapy including MTX given at the level of 3 g/m² on Day 14 of a cycle. Bleomycin, Adriamycin, cyclophosphamide, and vincristine are given on Day 1 of the cycle. Dexamethasone 6 mg/m² is given orally on Days 1 through 5. The cycle is repeated on Day 21, and patients are treated with 10 such cycles for a total of 30 weeks. Of our 42 patients who are evaluable for response and who have completed chemotherapy, 33 (78%) achieved a complete remission (CR). A total of seven patients have relapsed. Only one patient in the CR group has relapsed in the CNS after the cessation of chemotherapy.

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The majority of patients with non-Hodgkin's lymphoma (NHL) can be divided into two general prognostic categories. The favorable category group (1) includes those with nodular and diffuse well-differentiated lymphocytic lymphoma according to the Rappaport classification. The long natural history and pattern of involvement confining disease to the lymph nodes, liver, and bone marrow have made it difficult to assess the impact of therapy with curative intent. The unfavorable prognosis group (2) includes those patients with diffuse histiocytic or lymphocytic poorly differentiated lymphoma. In this group the tumors assume a much more malignant nature with a historical median survival of less than 2 years. However, these tumors have been shown to be quite responsive to intensive combination chemotherapy and these favorable results have had a major impact on survival. This is especially true not only in diffuse histiocytic lymphoma, but also in Burkitt's lymphoma (3). Amongst these two variants, intensive treatment has resulted in long-term cures in approximately 30% to 40% of cases (4). The remainder suffer progression or relapse of disease and die within a short

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period even though the majority of patients have some response to the treatment. The recurrence of disease between cycles of effective chemotherapy usually signals progressive refractoriness and early demise. In addition, a significant percentage of patients with diffuse histiocytic lymphoma develop central nervous system (CNS) invasion during progression of relapse of their disease, although it is rarely the sole site of recurrence (5–8).

Improved results with cytotoxic chemotherapy would require either further intensification of the doses or the addition of other cytotoxic agents whose myelotoxicity, if present, would limit the doses of other agents and increase the myelosuppressive hazards to the patient.

The demonstration that high doses of methotrexate (MTX) could be safely given if followed by citrovorum factor (CF) has led to expanded use of this agent for certain tumors (9). MTX is particularly attractive since the myelosuppressive effects can be aborted by the rescue agent. The extensive pharmacologic investigations with higher doses of MTX have permitted monitoring of the potential for toxicity by serial determination of plasma levels of the drug (10,11). Our initial studies demonstrated antitumor activity for high-dose methotrexate with citrovorum factor rescue (HDMTX/CF) in NHL of unfavorable histology (12). In addition, the response of CNS metastases either within the cerebrum or in the meninges confirmed the pharmacologic data that demonstrated cytotoxic concentrations of MTX in the spinal fluid of patients receiving the higher doses (13,14). This report brings our experience with HDMTX/CF as a single agent up to date and describes our preliminary experience with the use of HDMTX/CF in combination with the other cytotoxic agents: cyclophosphamide, bleomycin, vincristine, Adriamycin, and prednisone. An abstract initial treatment protocol has been previously reported (15).

MATERIALS AND METHODS

The initial evaluation of HDMTX/CF was undertaken in patients with advanced NHL of the unfavorable histologic type such as diffuse poorly differentiated lymphocytic (DPDL), histiocytic (DH), and undifferentiated lymphoma (DU). The initial series included 25 patients who, with one exception, had failed to respond to conventional therapy. The age, stage, and response to therapy are shown for each patient in table 1. Informed consent was obtained in all instances. It was required that each patient have a creatinine clearance in excess of 60 ml/minute and, in most instances, a normal IVP. Alkalinization of the urine was achieved by the administration of sodium bicarbonate 3 g orally every 3 to 6 hours on the night prior to and for 48 hours following the MTX infusion. Hydration was initiated with 3 liters of fluid/day and maintained. The drug was supplied by the Division of Cancer Treatment of the National Cancer Institute. Prior to infusion, the drug was dissolved in 5% dextrose and water, then it was administered as an iv infusion over a 20- to 40-minute period. The initial dose was 1 g/m² but was escalated in selected patients to between 3 and 7.5 g/m² if no toxicity was noted at the lower doses. CF was given at 10 mg/m² iv at 24 hours following the MTX and continued at that dosage level orally every 6 hours for 72 hours. If serum creatinine increased by more than 50% at 24 hours, the CF dose was increased to 100 mg orally every 3 hours until the serum MTX level decreased below 1×10^{7} M. The courses of MTX were initially given weekly, and in some instances were separated by 2- to 3-week intervals in those few patients who responded over long periods of time.

Patients were in either stage III or stage IV and in most instances had extensive involvement of bone marrow, liver, and gastrointestinal tract. Six patients had CNS involvement by tumor characterized by an elevation in spinal fluid protein. Four of the five patients demonstrated a positive spinal fluid cytology. Complete response (CR) was defined as complete disappearance of all measurable evidence of disease for at least 1 month. Partial response (PR) was defined as a greater than 50% reduction in the measurable lesions lasting for at least 1 month. The duration of response and survival were measured from the time of initiation of chemotherapy. Initially a minimum of three courses were administered to each patient. Serum MTX levels were determined by radioimmunoassay at 24 hours according to previously published methods (10). The pathologic classification of Rappaport was used. Of the 24 patients, 13 had DPDL and the remainder had DH. Two patients classified as DPDL had lymphosarcoma cell leukemia.

Fifty-four previously untreated patients with either DH or DU were treated with a combination chemotherapy program known as M-BACOD. All patients had advanced disease in stage III or stage IV. Staging evaluation included a bone marrow biopsy and evaluation of the retroperitoneum and, in some instances, an abdominal CT scan or abdominal ultrasound. Staging laparotomy was not utilized. All histopathologic slides were reviewed by a single team of hemapathologists. In this regimen bleomycin 4 mg/m², Adriamycin 45 mg/m², cyclophosphamide 600 mg/m², and Oncovin® 1 mg/m² were administered iv on Day 1. At that time dexamethasone was begun at 6 mg/m² and given orally over 5 days. On Day 14 the patient was given an infusion of MTX 3 g/m² over a 20- to 40-minute period as described above, regardless of the blood counts. The cycle of previously mentioned cytotoxic drugs was repeated on Day 21. Patients were scheduled to receive 10 cycles over 30 weeks. after which all therapy was stopped and they were reevaluated.

TABLE 1.—The effect of HDMTX/CF in previously treated patients with non-Hodgkin's lymphoma

Patient	Age	Sex	Histology	Stage	Extra nodal sites	Prior therapy	Response to MTX/FA (month)
1	55	M	DPDL	IV	CNS	BACOP, XRT	CR 2.0
2	27	F	DPDL	IV	Marrow	BACOP, XRT	CR 3.5
3	28	M	DPDL	IV	CNS	BACOP	CR 16.0
4	22	F	DH	III_E	Breast	COP, BACOP XRT	CR 13.0
5	50	F	DH	IV	CNS	XRT	CR 21.0
6	31	M	DH	IV	Marrow	COP, Chlorambucil.	PR 1.0
7	54	F	DPDL	IV	Marrow, liver	COP, BACOP	PR 6.0
8	34	M	DPDL	IV	Marrow	BACOP	PR 2.5
9	68	M	DPDL	IV	CNS, pleura	BACOP	PR 1.0
10	33	M	DPDL	IV	Skin, lung, marrow, blood.	COP	PR 1.0
11	34	M	DPDL	IV	Lung, liver, CNS	BACOP	PR 4.0
12	68	F	DH	IV	Skin	COP, A, XRT	NR
13	26	F	DH	IV	Lung, skin	BACOP, XRT	NR
14	54	M	DH	IV	Liver, marrow, CNS.	BACOP	NR
15	31	F	DH	IV	Liver	COP, BCNU	NR
16	33	M	DH	IV	Skin. marrow CNS	BACOP	NR
17	54	F	DPDL	IV	Liver, marrow, blood.	COP, BACOP, XRT.	NR
18	60	M	DPDL	IV	Marrow, blood	COP, Chlorambucil.	NR
19	61	M	DPDL, Leukemia	IV	Bone marrow, nodes.	BACOP	NR
20	70	F	DH	III		BACOP	PR 2.5
21	61	F	DH	IV	Liver, stomach, bone marrow, nodes.	BACOD	NR
22	67	M	DH	IV	Bone marrow, liver, intestinal, retroperitoneal.	BACOD	PR 1.0
23	62	F	DPDL	IV	Bone marrow, peripheral blood.	COP	NR
24	26	M	DH	IV	Lung	C-MOPP	NR

RESULTS

HDMTX/CF as a Single Agent in Previously Treated Patients

A median of six courses of MTX were administered to each patient, although a minimum of three cycles of therapy were initially required for evaluation. The demonstration that all responders showed some evidence of shrinkage in the first cycle suggested that even the response to a single course might be considered evaluable. Thus, one patient is included who had one cycle but had readily measurable disease in the lungs and failed to respond. Of 25 patients, 13 (52%) have demonstrated response to MTX. In five of these patients, there was complete disappearance of all evidence of disease. These sites included the bone marrow, the CNS in three patients, and breast mass. Seven patients responded from 1 to 2.5 months. In the remainder, the response duration was 4, 6, 13, 16, and 21 months. In all instances measurable disease was present. Of the six patients with CNS involvement, all but one responded. Responses included reversion of brain scan abnormalities toward normal (two patients). In two patients the response of CNS involvement lasted 16 and 21 months respectively. The toxicity of HDMTX has been previously described when used as a phase II agent (10). Three percent of courses resulted in mild renal insufficiency. Mucositis was noted in six courses. In the majority of the patients, the drug was well tolerated.

M-BACOD THERAPY

At the present time, 42 evaluable patients have been entered into the M-BACOD protocol, which began in January 1976. Of the 42 patients, 33 (78%) entered CR (table 2). Four patients achieved PR, and five had no response. Of the 33 patients who entered CR, 7 have had a relapse, all between 12 and 26 months following the initiation of therapy. The median duration of response at the present time is 16+ months. There have been no late relapses beyond 24 months. The toxicity of this program is

evaluable in 39 patients or 342 cycles of combination chemotherapy. Only 8% of courses had a wbc count below 1,000, but in the vast majority of cases, the nadir of the wbc count went below 4,000, with 28% of the courses down to the 2,000 to 3,000 range. Five percent had a bleomycin-related skin rash and 8 of 39 patients (21%) had what appeared to be bleomycin-related diffuse interstitial pulmonary pneumonitis. MTX-related mucositis was seen in only 4 of 342 cycles (1.2%). An increase in creatinine was noted in 7 of 342 courses (2%). There has been only one late CNS relapse in a CR patient who has stopped chemotherapy. Two patients developed CNS recurrence early in the course of their treatment and thus were not entered into the CR group. The six patients with bone marrow involvement who entered CR had remained free of CNS recurrence to date.

TABLE 2.—MTX in combination chemotherapy of non-Hodgkin's lymphoma—M-BACOD

Diffuse histiocytic/undifferentiated lymphoma (1/1/76–12/31/78)	
Total	42
CR	33
PR	4
None	5
Median duration	16+ months
Relapse	7

DISCUSSION

Courses of HDMTX/CF were capable of producing useful tumor regression in 52% of patients with diffuse NHL. The most encouraging aspect of these short-lived responses is the fact that CNS disease was responsive for a relatively short interval (12). Although serious toxicity was rare under careful observation, the expense and need for extensive hydration and monitoring of plasma levels do not justify the general use of HDMTX in the treatment of refractory lymphoma. The one exception might be primary histiocytic lymphoma of the brain, which is an exceedingly rare variant but quite responsive to MTX/CF (16,17). The marked antitumor effect and lack of significant myelotoxicity prompted its inclusion in de novo combination chemotherapy since, in this setting, HDMTX/CF can contribute antitumor activity during periods of drug-induced myelosuppression when no other myelosuppressive agents can be given. An attempt at treatment with nonmyelosuppressive therapy with bleomycin and vincristine did not appear to increase the long-term disease-free survivorship in DH as compared to earlier successful programs such as (C) MOPP (18).

The preliminary results of the M-BACOD program appear gratifying, with a high CR rate, in the range of 80%. The extent of these remissions has been confirmed by repeat staging procedures that, in the last 2 years, have included abdominal computerized tomography. The relapse rate at the present time is 20%, which is somewhat less than for previously reported regimens (1). The fact that only one patient achieving CR developed CNS recurrence may represent a unique accomplishment of the M-BACOD program. Thus far, none of the patients in the CR group with bone marrow involvement have had a CNS relapse. It is well known that those patients with bone marrow involvement have a higher frequency of CNS relapse (5,6). In fact, the observed incidence of CNS relapse in patients with DH and bone marrow involvement in three series is 25 of 85 cases (about 30%) (6–8). In the majority of instances CNS relapse accompanies systemic dissemination, but in 13% to 26% of cases it occurred as the first sign of recurrence.

All sites of metastatic involvement appear responsive to this program. The occurrence of CNS relapse shortly after the initiation of therapy in two patients would suggest that metastatic involvement was already present prior to the initiation of therapy. The ultimate value of HDMTX/CF as a prophylactic agent against CNS relapse must await later analysis, especially in patients with bone marrow involvement. It is also noteworthy that in the vast majority of patients, HDMTX/CF was given on Day 14 following cyclophosphamide, Adriamycin, bleomycin, and prednisone or dexamethasone without exaggerating the myelosuppression or delay in retreatment by Day 21. If the present results continue, approximately 60% to 65% of patients presenting with DU or DH may be rendered free of disease for prolonged periods of time, perhaps indefinitely. The inclusion of HDMTX/CF with other agents has not received extensive trial (13,14,19). The COMA program includes MTX/CF with cytosine arabinoside following cyclophosphamide and vincristine. A preliminary evaluation indicates that it has been successful in the induction of remission (19). It does not include Adriamycin and includes prolonged administration of the antimetabolites on a weekly basis over 8 weeks. The latter, as well as the M-BACOD program, employs the principle of using antimetabolites following initial treatment with vincristine, cyclophosphamide, and, in M-BACOD, Adriamycin. It is postulated that residual tumor cells recovering from the effects of these agents would be likely to be in synchrony with an increased fraction of cells in DNA synthesis. The cytotoxic efficacy of the M-BACOD program may result from these kinetic phenomena and the ability of MTX/CF to penetrate the CNS.

REFERENCES

1. Canellos GP, et al. Chemotherapy of the non-Hodgkin's lymphomas. Cancer 42:932–940, 1978.
2. Portlock CS, et al. Treatment of advanced non-Hodgkin's lymphomas with favorable histologies: preliminary results of a prospective trial. Blood 47:745–756, 1976.
3. Fisher RI, et al. Prognostic factors for advanced diffuse histiocytic lymphoma following treatment with combination chemotherapy. Am J Med 63:177–182, 1977.
4. Arseneau JC, et al. American Burkitt's lymphoma: a clinicopathologic study of 30 cases. Am J Med 5:314–329, 1975.
5. Herman TS, et al. Involvement of the central nervous system by non-Hodgkin's lymphoma. Cancer 43:390–397, 1979.
6. Young RC, et al. Central nervous system complications of non-Hodgkin's lymphoma: the potential role for prophylactic therapy. Am J Med 66: 435–449, 1979.
7. Levitt LJ, et al. CNS involvement in the non-Hodgkin's lymphomas. Cancer 45:545–552, 1980.
8. Litam JP, et al. Central nervous system relapse in malignant lymphomas: risk factors and implications for prophylaxis. Blood 54:1249–1257, 1979.
9. Frei E (III), et al. New approaches to cancer chemotherapy with methotrexate. N Engl J Med 292:846–851, 1975.
10. Stoller RG, et al. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. N Engl J Med 297:630–634, 1977.
11. Nirenberg A, et al. High-dose methotrexate with citrovorum factor rescue: predictive value of serum methotrexate concentrations and corrective measures to avert toxicity. Cancer Treat Rep 61:779–783, 1977.
12. Skarin AT, et al. High-dose methotrexate with folinic acid in the treatment of advanced non-Hodgkin's lymphoma including CNS involvement. Blood 50:1039–1047, 1977.
13. Tattersall MHN, et al. Clinical pharmacology of high-dose methotrexate (NSC-740). Cancer Chemother Rep 6:25–29, 1975.
14. Shapiro WR, et al. Methotrexate: distribution in cerebrospinal fluid after intravenous, ventricular and lumbar injections. N Engl J Med 293:161–166, 1975.
15. Skarin A, et al. Therapy of unfavorable histology non-Hodgkin's lymphoma (NHL) with high dose methotrexate and citrovorum factor rescue (MTX/CF), bleomycin (b), Adriamycin (A), cyclophosphamide (C), Oncovin ® (O), and decadron (D) (M-BACOD). Proc Am Assoc Cancer Res and ASCO 19:400, 1978.
16. Herbst KD, et al. Successful therapy with methotrexate of a multicentric mixed lymphoma of the central nervous system. Cancer 38: 1476–1478, 1976.
17. Henry JM, et al. Primary malignant lymphomas of the central nervous system. Cancer 34:1293–1302, 1974.
18. Schein PS, et al. Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. Ann Intern Med 85:417–422, 1976.
19. Berd D, et al. Long-term remission in diffuse histiocytic lymphoma treated with combination sequential chemotherapy. Cancer 35:1050–1054, 1975.

Clinical Use of Methotrexate—With Emphasis on Use of High Doses [1]

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SUMMARY

The rationale for and the clinical use of regimens that utilize methotrexate (MTX) in high dose (>500 mg/m²) are reviewed. Advantages of high pulse doses are (a) increased cell kill; (b) prevention or delay of resistance; (c) penetration into the cerebrospinal fluid (CSF); and (d) relative lack of toxicity, if used with appropriate monitoring and patient surveillance. Disadvantages are its cost and the cost of patient followup required. Some examples of its use in combination illustrate the point that antagonistic as well as synergistic antitumor or antinormal cell effects can result, depending upon the sequence of administration of MTX and another drug.

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During the past decade, the clinical use of methotrexate (MTX) to treat neoplastic disease has undergone a sort of renaissance. Based upon the leads afforded by Goldin and coworkers (1,2), various dose schedules with and without the MTX antidote leucovorin (LV) (N^5-formyl tetrahydrofolate, citrovorum factor) have been evaluated (table 1). These various dose schedules and a continued escalation of MTX dosage with LV rescue have led to a great deal of confusion and difficulty on the part of practicing physicians using this drug. In this brief report, the advantages, problems, and clinical use of MTX, especially in high-dose regimens, will be reviewed.[4]

TABLE 1.—Dose schedules of MTX

Dose	Route	Frequency	LV
I. Conventional dose			
(a) 15 to 20 mg/m²	po or iv	2 × week	–
(b) 30 to 50 mg/m²	po or iv	weekly	–
(c) 15 mg/m² × 5 days.	iv or im	q 2 to 3 weeks	–
II. Moderate dose			
(a) 50 to 150 mg/m²	iv push	q 2 to 3 weeks	–
(b) 240 mg/m²	iv infusion (24 hours).	q 4 to 7 days	+
(c) 0.5 to 1.0 g/m²	iv infusion (36 to 42 hours).	q 2 to 3 weeks	+
III. High dose			
(a) 1 to 12 g/m²	iv (1 to 6 hours)	q 1 to 3 weeks	+

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Advantages of High-Dose, Intermittent Pulses of MTX

Goldin et al (1) have established that intermittent pulses of MTX, which can be escalated to high doses by administering LV 12 or 24 hours after the antifolate, are more effective than daily low-dose treatment in the early phases of growth (logarithmic growth) of the rapidly growing transplantable neoplasm, L1210. These studies have been extended to the clinic, and intermittent therapy with high doses of MTX without LV in patients with acute lymphoblastic leukemia (ALL) in remission was shown to give a longer remission duration than low-dose daily administration of the antifolate (6). Thus it would also follow that high doses of MTX, given in pulse fashion with LV rescue, might be even more effective treatment of ALL patients in remission. However, certain patients in these studies have developed leukoencephalopathy, which is associated with previous cranial irradiation (5). It would seem that the advantage of high pulse doses of MTX would be in the treatment of rapidly growing neoplasms in their rapid phase of growth (eg, treatment in remission, adjuvant therapy). Since other factors in addition to growth rate influence MTX sensitivity, it also follows that this considera-

[4] For other recent reviews of this subject, see Bertino (3), Chabner and Johns (4), and Bleyer (5).

tion applies to tumors with some degree of intrinsic sensitivity.

A second advantage of high-dose regimens of MTX is the prevention or delay of drug resistance. In experimental tumor models, both in vitro and in vivo, resistance to MTX occurs more rapidly when small doses of the drug are administered continuously (7,8). In some experimental situations, as well as in the treatment of human malignancy, resistance to low doses of MTX may be overcome by the use of large doses of the antifolate, usually requiring LV rescue (9,10). Success of this maneuver may depend on the mechanism of resistance—if MTX transport is deleted (probably a rare cause of in vivo MTX resistance), very large doses of MTX may not be able to result in significant intracellular drug levels to inhibit cell growth. Large pulses of MTX also may be of value in the treatment of tumors refractory to conventional doses of MTX; this could be due to one of several reasons: (a) poor active transport of MTX, but some passive diffusion at high levels; (b) the ability of a cell to accumulate MTX (probably as polyglutamates), which then prevents replication, even in a population that is not rapidly dividing; and (c) biochemical effects of MTX or its products (or its impurities) in cells that affect other targets in addition to dihydrofolate reductase (DHFR). One clinical example that illustrates this point is the treatment of osteosarcoma—all the available data indicate that very high doses of MTX (>8 g/m^2) are required for antitumor effects (11).

Despite these theoretical considerations, resistance to high-dose MTX with LV rescue can occur rapidly, as we have noted in the treatment of some human malignancies, eg, head and neck cancer (12), and acute nonlymphocytic leukemia (10). In this circumstance, resistance may not be due to the emergence of a mutant population with an increased DHFR, etc, but may reflect accumulation of reduced folates as a consequence of LV administration. Thus if LV is given too early, or in excess, the tumor, as well as the normal renewal tissues, may be spared.

A third advantage of high-dose MTX regimens, somewhat unanticipated, is the cytocidal concentrations achieved in the cerebrospinal fluid (CSF). Doses of >500 mg/m^2, given as either a short-term (1 hour) or long infusion (24 to 42 hours), give drug levels in the CSF of $>10^{-7}$ M (13); at these concentrations DNA synthesis in leukemia cells is markedly inhibited (10). Thus it is possible to treat patients with meningeal leukemia, lymphoma, or even carcinomatosis with high-dose infusions of MTX with LV rescue, although experience with this approach is limited (14). Brain tumors also have been treated with high-dose regimens of MTX, with equivocal results; penetration of MTX into the depths of a tumor may be difficult to achieve with this polar molecule (15). Another approach to the treatment of brain tumors is the use of a folate (and MTX) cleaving enzyme, carboxypeptidase G_1. Abelson et al have administered this enzyme 24 hours after high-dose MTX—this enzyme inactivates MTX in the plasma, but not in the CSF—thus toxicity to peripheral tissues is prevented while high levels in the CSF are maintained (16).

One additional benefit of high-dose MTX, and perhaps the most important advantage of using LV rescue, is the relative lack of toxicity of MTX if used according to guidelines recently established (vide infra). The relative lack of marrow toxicity of this regimen had led to the incorporation of high-dose MTX into many combination regimens. As will be discussed below, such combinations may be antagonistic as well as synergistic, and therefore MTX should be used in these circumstances only if experimental or clinical evidence indicates that some therapeutic benefit might accrue.

The disadvantages of high-dose regimens also should be mentioned; in addition to the cost of the drug, patients must be carefully monitored and followed, adding to the overall expense of the program (17,18).

Clinical Use of High-Dose Regimens

Guidelines for the use of MTX, based upon the experience of this center, as well as others, are given in tables 2 and 3. Questions not completely resolved are the optimum MTX dose, the duration of the infusion, and the frequency of dosing. It is also clear that the LV dose and duration of rescue should be a function of the MTX blood level at the time LV is instituted, since this reduced folate competes with MTX for uptake and bypasses the MTX-induced block of DHFR as well (7). Experimental evidence has been presented that supports the contention that too much LV may decrease MTX antitumor effects (19). A rational LV rescue schedule based on MTX plasma levels has been suggested by Jacobs and Santicky (20) on the basis of clinical trials and is given in table 3. While infusions of MTX of 6 to 42 hours are an excellent way of obtaining a desired blood level over a period of time, they have the disadvantage of usually requiring inpatient care. Alternate schedules have been utilized (3 to 7 g/m^2 over 30 to 60 minutes), which give 24-hour levels of MTX in most patients of $>10^{-6}$ M, and may be repeated at weekly intervals on an outpatient basis (17).

By appropriate monitoring of the serum creatinine and plasma MTX levels (table 2), high-dose MTX administration in experienced hands has proven to be relatively safe and nontoxic (17,21). The cause of the renal toxicity observed in the high-dose regimens is presumably due to nephropathy resulting from MTX or its 7-OH metabolite (22) precipitating in the renal tubules, but a direct toxic effect of MTX on renal tubules, even with lower doses, can occur (23). In both circumstances the renal effects are usually reversible, but unless appropriate action is taken to improve elimination of the drug and to remedy the effects of high concentrations of MTX that result, patients will

succumb to the consequences of hematologic and gastrointestinal toxicity. A major problem exists when patients develop renal failure after high-dose MTX. Dialysis, either alone or with charcoal, has not been useful because of the large tissue stores that replenish the plasma when dialysis stops (24). In addition, MTX does not dialyze well (25). Carboxypeptidase G_1 has been used with success in lowering MTX levels (25,26), but the product of this enzymic inactivation of MTX is 2,4-diamino-N^{10}-methylpteroic acid, even less soluble than MTX in urine. In some circumstances weeks of LV therapy are necessary with close monitoring of blood levels.

TABLE 2.—Guidelines for high-dose MTX therapy with LV rescue

1. A normal creatinine clearance should be required before therapy. A normal serum creatinine should be required before each additional course of therapy.
2. Alkalinize the urine before and during the MTX therapy with po sodium bicarbonate to pH 7.0 or higher.
3. Push fluids to 3,000 ml/m²/day during the 24 hours following MTX administration.
4. Obtain a serum creatinine and plasma or serum MTX 24 hours after starting MTX.
   (a) If serum creatinine is >50% elevated above pretreatment levels, increase LV to 100 mg q 6 hours and continue until plasma MTX $<10^{-8}$ M.
   (b) Adjust LV rescue according to plasma MTX (table 3).

TABLE 3.—LV rescue schedules following high-dose MTX*

Plasma level at 24 to 30 hours**	LV Dose	Duration
$< 1.5 \times 10^{-6}$ M	10 to 15 mg/m² q 6 hours.	48 hours
1.5 to 5.0×10^{-6} M	30 mg/m² q 6 hours	until plasma level $<5 \times 10^{-8}$ M
$> 5.0 \times 10^{-6}$ M	60 to 100 mg/m² q 6 hours.	until plasma level $<5 \times 10^{-8}$ M

*Modified from Jacobs and Santicky (20).
**After iv bolus or infusion therapy.

Despite the large number of dose schedules employed, additional work is necessary to establish optimum dosage schedules, which probably will be different for each tumor. It seems unlikely that we will ever achieve this goal, given the fact that most human tumors are heterogeneous (eg, head and neck cancer), and in the present era of combination chemotherapy, single agents are rarely used.

Intrathecal Use

After an intrathecal injection, most patients clear MTX from their CSF within 7 days of injection, and neurotoxicity is rare if drug administration is at intervals of 7 days or more (5). If MTX is given more frequently, monitoring of spinal fluid levels with appropriate dose modification can reduce the frequency of neurotoxicity. Bleyer (5) has given guidelines for CSF levels of MTX in patients treated with intrathecal MTX that may be used to estimate subsequent dose modifications. Bleyer (5) has also pointed out that the 12 mg/m² conventional dose (maximum 15 mg total dose) was not adequate to prevent meningeal leukemia in children less than 3 years of age, and that body surface measurements do not correlate well with CSF levels of MTX obtained after the 12 mg/m² dose. Table 4 indicates the dosage schedule he suggests for intrathecal MTX; note that all patients over the age of 3 receive 12 mg (total) of MTX, regardless of body surface area.

TABLE 4.—Guidelines for intrathecal therapy

Age (years)	Total MTX dose (mg)
< 1	6
1	8
2	10
>3	12

Neurotoxicity because of MTX can occur following intrathecal MTX or with systemic administration of large doses (> 80 mg/m²).

An acute syndrome consisting of headache, fever, meningismus, and a pleocytosis that may mimic bacterial infection has been described following intrathecal administration. This syndrome is the most common of the neurologic syndromes associated with intrathecal MTX, and appears to be related to high CSF concentration of MTX (5).

A subacute form of CNS toxicity also has been described following intrathecal use, and includes more severe signs of motor dysfunction of the brain such as paraplegia, cerebellar dysfunction, cranial nerve palsies, and seizures, usually following two or more doses of drug per week. This complication has been correlated with persistently elevated levels of MTX in the CSF.

In addition to the neurotoxicity reported above after intrathecal MTX, patients receiving high-dose MTX (> 50 mg/m²) parenterally after radiation therapy to the brain (> 2,000 rad) have developed a necrotizing leukoencephalopathy associated with progressive neurologic deterioration. This may begin insidiously and progress to severe neurologic impairment (dementia, ataxia, seizures, and coma). Bleyer has pointed out that in the 58 cases reported thus far, most were treated with combinations of either systemic or intrathecal MTX (or both) with cranial irradiation (5).

Combination Chemotherapy That Includes High-Dose MTX

As mentioned earlier, a major advantage of high-dose MTX regimens is the relative lack of major toxicities, especially marrow and gastrointestinal. As a consequence, it is perhaps not surprising that high-dose MTX has been incorporated into many treatment programs as part of drug combinations. The value of MTX administered as part of these regimens is often difficult, if not impossible, to evaluate, and it is possible that the antitumor effects of the combination might be lessened in some combinations and enhanced in others by the inclusion of MTX in high dosage. To illustrate this point, some of the Yale experimental and clinical experience with MTX used in combination with other drugs is discussed below.

MTX and L-asparaginase

Earlier studies by Capizzi et al (27) in experimental systems established that asparaginase, when given together with MTX, could abolish MTX antitumor effects. However, by delaying asparaginase administration for 24 hours, it was possible in experimental systems, as well as in patients with ALL, to obtain synergistic effects with this combination, even in patients previously treated with lower doses of MTX. Asparaginase, although toxic to lymphoid neoplastic cells (T- and Null cells), was essentially nontoxic to normal stem cells, and in fact antagonized MTX effects by inhibiting protein synthesis, and may be considered an MTX rescue agent. It has been possible to give up to 600 mg/m² of MTX at 10-day intervals with little or no marrow toxicity, if asparaginase is given 24 hours later (28). It may be possible to increase the tolerated dose with additional LV rescue, but the value of this approach has not been explored fully.

MTX and Cytosine Arabinoside (Ara-C)

This combination also appears to be sequence dependent in experimental tumors (29,30). Our studies in mice bearing the L1210 tumor,[5] as well as those of Avery and Roberts (29), indicated that the drugs were most effective when given simultaneously, or when MTX was given a short time prior to Ara-C. The combination is of interest in that additive toxicity was not observed in marrow stem cells (S-CFU), but additive and synergistic activity was obtained with this combination on lymphoma stem cells (30). Recently Cadman et al (31) have shown that MTX pretreatment results in enhanced Ara-CTP formation, the presumed effective intracellular antimetabolite. We have used the combination of moderate-dose MTX followed by Ara-C as the second part of protocols for the treatment of diffuse histiocytic lymphoma, presumably when tumor cells are in log phase growth and most susceptible to antimetabolite effects (3).

[5] Unpublished observations.

MTX and Fluorouracil (FU)

The rationale for the use of these drugs in the sequence MTX-FU with a 1- to 4-hour interval between the drugs is developed more fully by Fernandes et al (32). Based upon these and other studies, clinical protocols have been developed that will test the hypothesis that sequential use of MTX and FU is more effective than the use of this combination given simultaneously.

MTX and cis-Platinum Diammine Dichloride (DDP)

Since both of these agents are effective in the treatment of squamous cell carcinoma, the use of this combination would therefore seem to be worthwhile. The problem in this instance, however, is not drug antagonism of tumor cell kill, but the possibility that these two potentially nephrotoxic substances would have additive kidney toxicity. In a study just completed by Pitman and coworkers at Yale (33) in patients with cancer of the head and neck, the sequential use of DDP (80 mg/m² q 3 weeks × 2), followed by weekly pulses of high-dose MTX (3 g/m² q week × 4) proved to be nephrotoxic, thus decreasing the amount of MTX that could be subsequently administered. The opposite sequence, namely MTX first, followed by DDP, at least allowed the administration of MTX in full dose, followed by DDP in full dose for most patients.

CONCLUSIONS

It should be clear from this brief review that, despite a large body of information dealing with the use of MTX, this topic is still deserving of further careful study. A plea is made for controlled studies whenever possible, so that the value of high doses of this drug can be determined. Other rescue techniques, such as the use of carboxypeptidase G_1 and thymidine, also deserve further exploration.

REFERENCES

1. Goldin A, Venditti JM, Kline I, et al. Eradication of leukemia cells (L1210) by methotrexate and methotrexate plus citrovorum factor. Nature 212:1548-1550, 1966.
2. Goldin A, Venditti JM, Humphreys SR, et al. Studies on the management of mouse leukemia (L1210) with antagonists of folic acid. Cancer Res 15:742-747, 1955.
3. Bertino JR. "Rescue" techniques in cancer chemotherapy: use of leucovorin and other rescue agents after methotrexate treatment. Semin Oncol 4:203-216, 1977.
4. Chabner B, and Johns D. Folate antagonists. *In* Cancer—A Comprehensive Treatise. Chemotherapy (Becker F, ed). 1977, vol V, pp 363-377.
5. Bleyer WA. Methotrexate: clinical pharmacology, current status and therapeutic guidelines. Cancer Treat Rev 4:87-101, 1977.
6. Acute Leukemia Group B. New treatment schedule with improved survival in childhood leukemia. Intermittent parenteral

vs daily oral administration of methotrexate for maintenance of induced remission. JAMA 194:75, 1965.
7. Bertino JR. Toward improved selectivity in cancer chemotherapy: the Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res 39:293–304, 1979.
8. Schrecker AW, Mead JAR, Greenberg NH, et al. Dihydrofolate reductase activity of leukemia L1210 during development of methotrexate resistance. Biochem Pharmacol 20:716–718, 1971.
9. Djerassi I, Abir E, and Roger GL (jr). Long term remission in childhood acute leukemia: use of infrequent infusions of methotrexate: supportive role of platelet transfusions and citrovorum factor. Clin Pediatr 5:502–509, 1966.
10. Hryniuk WM, and Bertino JR. Treatment of leukemia with large doses of methotrexate and folinic acid: clinical-biochemical correlates. J Clin Invest 48:2140–2155, 1969.
11. Rosen G, Murphy ML, Huvos AG, et al. Chemotherapy, in block resection, and prosthetic bone replacement in the treatment of osteogenic sarcoma. Cancer 37:1, 1976.
12. Bertino JR, Mosher MB, and DeConti RC. Chemotherapy of cancer of the head and neck. Cancer 31:1141–1149, 1973.
13. Shapiro WR, Young DF, and Mehta BM. Methotrexate: distribution in cerebrospinal fluid after intravenous, ventricular and lumbar injections. N Engl J Med 293:161–166, 1975.
14. Skarin AT, Zuckerman KS, Pitman, SW, et al. High dose methotrexate with folinic acid in the treatment of advanced non-Hodgkin's lymphoma including central nervous system involvement. Blood 50:1039–1048, 1977.
15. Rosen GA, Ghavimi F, Nirenberg A, et al. High dose methotrexate with citrovorum factor rescue for the treatment of central nervous system tumors in children. Cancer Treat Rep 61:681–690, 1977.
16. Abelson H, Ensminger W, Rosawsky A, et al. High-dose methotrexate-carboxypeptidase G_1—A selective approach to the therapy of central nervous system tumors. *In* Chemistry and Biology of Pteridines, VIth International Pteridine Symposium. (Brown GM, ed). New York, NY, Elsevier, 1979.
17. Pitman SW, and Frei E (iii). Weekly MTX-calcium-leucovorin rescue: effect of alkalinization on nephrotoxicity; pharmacokinetics in the CNS; and use in CNS non-Hodgkin's lymphoma. Cancer Treat Rep 61:695, 1977.
18. Stoller RG, Hande RK, Jacobs SA, et al. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. N Engl J Med 297:630–634, 1977.
19. Sirotnak FM, Moccio DM, and Dorick DH. Optimization of high-dose methotrexate with leucovorin rescue therapy in the L1210 leukemia and sarcoma 180 murine tumor models. Cancer Res 38:345–353, 1978.
20. Jacobs SA, and Santicky MJ. Phase I trial of high-dose methotrexate with modified citrovorum factor rescue. Cancer Treat Rep 62:397–399, 1978.
21. Isacoff WH, Eilber F, Tabbarah H, et al. Phase II clinical trial with high-dose methotrexate therapy and citrovorum factor rescue. Cancer Treat Rep 62:1295–1304, 1978.
22. Jacobs SA, Stoller RG, Chabner BA, et al. 7-OH methotrexate as a urinary metabolite in human subjects and rhesus monkeys receiving high dose methotrexate. J Clin Invest 57:534–538, 1976.
23. Condit PT, Chanes RE, and Joel W. The renal toxicity of methotrexate. Cancer 23:126–131, 1969.
24. Djarassi I, Kim JS, and Shulman K. High dose methotrexate-citrovorum factor rescue in the management of brain tumors. Cancer Treat Rep 61:691–694, 1977.
25. Howell SB, Blair HE, Uren J, et al. Hemodialysis and enzymatic cleavage of methotrexate in man. Eur J Cancer 14:787–792, 1978.
26. Bertino JR, Skeel RT, Makulu D, et al. Initial clinical studies with carboxypeptidase G_1 (CPG_1), a folate depleting enzyme. Clin Res 483A, 1974.
27. Capizzi RL, Summers WP, and Bertino JR. L-asparaginase induced alteration of amethopterin (methotrexate) activity in mouse leukemia L5178Y. Ann NY Acad Sci 186:302–311, 1971.
28. Capizzi RL. Improvement in the therapeutic index of methotrexate by asparaginase. Cancer Chemother Rep 6(part 3):37–41, 1975.
29. Avery TL, and Roberts D. Dose-related synergism of cytosine arabinoside and methotrexate against murine leukemia L1210. Eur J Cancer 10:425–429, 1974.
30. Edelstein M, Vietti T, and Valeriote F. The enhanced cytotoxicity of combinations of 1_β–o arabinofuranosylcytosine and methotrexate. Cancer Res 35:1555–1558, 1975.
31. Cadman EC, and Eiferman F. Enhancement of Ara-C antitumor activity by methotrexate: a biochemical study. J Clin Invest, 1979. In press.
32. Fernandes DJ, Moroson BA, and Bertino JR. The role of methotrexate and dihydrofolate polyglutamates in the enhancement of fluorouracil action by methotrexate. Cancer Treat Rep 65 (Suppl. 1):29–35, 1981.
33. Pitman SW, Minor DR, Papac R, et al. Sequential methotrexate-leucovorin (MTX-LCV) and cis-platinum (CDDP) in head and neck cancer. Proc Am Assoc Cancer Res, 1979. In press.

Treatment of Central Nervous System Tumors with Methotrexate [1]

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SUMMARY

Thirteen patients with primary and metastatic CNS tumors have been treated with methotrexate (MTX) using three different approaches: (a) high-dose MTX with leucovorin (LV) rescue; (b) high-dose MTX with carboxypeptidase (CPDG) rescue; and (c) intraventricular administration of low doses of MTX for extended periods (concentration × time [C×T]). Eleven patients had central nervous system (CNS) lymphoma (one primary), one patient had recurrent medulloblastoma, and another patient had metastatic breast carcinoma. All 13 patients received high-dose MTX-LV rescue, while 3 patients were subsequently given MTX-CPDG. One patient received MTX by all three modalities. In patients with CNS lymphomas, complete responses (45%) and partial responses (36%) produced CNS disease-free intervals ranging from 1 to 23+ months. Survival for the complete responders has thus far ranged from 2.5 to 35 months, while the partial responders survived from 3 to 5 months. Two patients failed to respond and survived 2.5 and 3 months. Responses were obtainable with high-dose MTX-CPDG in patients resistant to MTX-LV. One patient who became sensitized to CPDG subsequently responded to MTX by intraventricular C×T administration.Thus, MTX can be effectively administered to patients with CNS tumors by several different approaches.

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The use of high-dose methotrexate (MTX) for the treatment of central nervous system (CNS) malignancies has met with variable success (1–5). It is clear from these studies, however, that MTX administered in high-dose protocols is able to penetrate the blood-cerebrospinal fluid (CSF) barrier, with peak CSF MTX levels exceeding 10^{-5} M (1–3,5).

In contrast to the limited usage of high-dose MTX, intrathecal or intraventricular MTX administration for the prophylaxis or treatment of meningeal leukemia and carcinomatosis, either alone or in combination with other drugs or with radiation therapy, is well established (6–8). Intrathecal MTX has also been used for the treatment of parenchymal brain tumors (9–11). Further, CSF MTX concentrations can be maintained in a "therapeutic" range with repeated MTX installation using the intraventricular route (6).

This report updates part of our experience using MTX for the treatment of primary and metastatic brain tumors using three different approaches: high-dose MTX with leucovorin (LV) rescue, high-dose MTX with carboxypeptidase (CPDG) rescue, and intraventricular administration of low doses of MTX for extended periods (concentration × time [C×T]).

MATERIALS AND METHODS

Eleven patients had CNS lymphoma; one had recurrent medulloblastoma, and another had CNS metastases of a breast adenocarcinoma. The clinical and pathologic characteristics of these patients are shown in table 1.

Prior to each course of high-dose MTX treatment, patients were required to have a normal serum creatinine and a creatinine clearance of > 50 ml/minute/m². Alkalinization of urine, hydration, MTX administration (1 to 7.5 g/m² over 20 to 40 minutes), LV rescue (10 mg/m² beginning 24 hours after the MTX and continued at 10 mg/m² every 6 hours for 72

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hours), and criteria for response were as previously described (5), except in case 6, in which a different MTX dose and schedule of administration were used (12). MTX levels were determined by radioimmunoassay (RIA) after LV rescue (13).

Three patients (cases 2, 6, 7) also received high-dose MTX (7.5 or 10 g/m²) followed by CPDG rescue. After CPDG rescue, MTX levels were determined by high pressure liquid chromotography (HPLC) rather than by RIA, since the CPDG cleavage product is recognized by the RIA antibody (12). Details of the HPLC procedure have been presented elsewhere (14).

One patient (case 7) also received treatment by C×T intraventricular MTX administration. MTX was given to this patient as a 6 mg loading dose into an Ommaya reservoir, and then 2 mg every 12 hours for a total of 5 to 7 days. Details of this procedure will be presented elsewhere.[5]

loblastoma (12) are updated. Toxicity associated with MTX treatment in these patients has been described elsewhere (5,12).

Of the 13 patients listed in table 1, 11 had non-Hodgkin's lymphoma of unfavorable histology. Of these 11 patients, 5 had complete responses for 2 to 23+ months (45%) and 4 had partial responses for 1 to 4 months (36%), for an overall response rate of 81% following high-dose MTX-LV therapy. Two patients showed no response to high-dose MTX-LV. Those patients receiving a minimum of three courses of high-dose MTX-LV were considered evaluable. Two additional patients with metastatic CNS lymphoma received only one or two doses of MTX-LV and were excluded because of rapid systemic disease progression.

Additional details are presented for four of the patients who illustrate the various therapeutic MTX approaches (table 1).

TABLE 1.—MTX therapy of CNS tumors

Patients	Age	Sex	Histology [1]	Systemic disease	Clinical findings	CSF cytology [2]	CNS response to MTX (months)	Survival after MTX (months)
1	20	F	DPDL	–	Headache, footdrop, 4+ papilledema.	+	CR 23+	24+
2	50	F	DH*	–	Headache, CT positive.	ND	CR 18	26+ [3]
3	55	M	DPDL	–	Headache, back pain.	+	CR 2	11
4	28	M	DPDL	+	Headache, cranial nerve palsy.	+	CR 16	35
5	54	M	DH	+	Headache, confusion, CT positive.	+	CR 2	2.5
6	13	M	Medulloblastoma	+	Headache.	+	CR 6	13 [3]
7	39	F	Adeno Ca breast	–	Headache, confusion, CT positive.	ND	PR 11	26+ [3,4]
8	71	F	DU	+	Leg weakness, incontinence.	+	PR 1	3
9	68	M	DPDL	+	Headache, confusion, leg paralysis.	+	PR 1	3.5
10	34	M	DPDL	+	Headache, seizure, CT positive.	ND	PR 4	4.5
11	29	M	DU	+	Headache, cranial nerve palsy.	+	PR 4	5
12	33	M	DH	+	Headache, cranial nerve palsy.	–	NR	3
13	66	F	DPDL	+	Headache, confusion.	+	NR	2.5

*Primary CNS lymphoma.
[1] DPDL, diffuse poorly-differentiated lymphocytic lymphoma; DH, diffuse histiocytic lymphoma; DU, diffuse undifferentiated.
[2] +, Malignant cells present; –, malignant cells absent; ND, not done.
[3] Also received MTX-CPDG.
[4] Also received intraventricular MTX by C×T.

RESULTS

Table 1 lists the histopathology, clinical findings, CSF cytology, CSF response to MTX, and survival after MTX for patients undergoing treatment. The previously reported survival data for eight patients (cases 2, 3, 4, 5, 8, 9, 10, 12) with CNS non-Hodgkin's lymphoma (5, 15) and one patient (case 6) with medul-

The first patient (case 1) has remained disease free for 23+ months. This patient's lymphoma was located in the cecum and terminal ileum. After biopsy, a side-to-side ileosigmoidostomy was performed and cyclophosphamide, vincristine, and prednisone (COP) were administered every 3 weeks for a total of four courses. Subcutaneous nodules were subsequently noted and an IVP revealed obstruction of the left ureter. Adriamycin (60 mg/m²/dose) was then given to a cumulative dose of 480 mg/m². This therapy

[5] Abelson HT, Kufe DW, Major P, et al. C×T therapy with methotrexate for central nervous system malignancies, in preparation.

resulted in resolution of both the subcutaneous nodules and the left ureteral obstruction. One month later, 9 months after the initial diagnosis, a CNS recurrence was noted with florid papilledema, fundal hemorrhages, partial loss of vision, right foot drop, and positive cytology. High-dose MTX, 3 g/m², was begun on a weekly basis × 6, with complete resolution of clinical symptomatology and CSF findings after five courses. MTX was then continued at the same dose every 3 to 4 weeks for 19 additional courses. Shortly after the last dose of MTX, the patient developed right lower quadrant pain and an exploratory laparotomy was performed that showed no evidence of recurrent disease. The patient has been off therapy now for 3 months and continues to be free of disease.

The second patient (case 2) (15) had a primary CNS lymphoma. She was initially treated with whole brain radiotherapy and corticosteroids. Because of subsequent disease progression, she was begun on high-dose MTX, 3 g/m² weekly for 10 weeks. There was rapid complete resolution of the CNS lesions that lasted for 18 months. At that time there was recurrence of headache and tumor was again noted on CT scan. The patient was retreated with MTX, but the dose was increased to 10 g/m², and rescue was with CPDG. After two administrations of high-dose MTX-CPDG rescue, tumors in both the right frontal and posterior fossa had decreased more than 50% in size. However, treatment could not be continued with MTX-CPDG since the patient developed an inactivating antibody to CPDG. Therapy was therefore begun again with MTX (3 g/m²) followed by LV rescue, but without response.

The third patient (case 6) (12) had recurrent medulloblastoma with widespread systemic bone disease and solid sheets of tumor cells in his CSF. Previous treatment consisted of radiotherapy on two separate occasions. The patient responded rapidly to MTX-LV with disappearance of tumor cells from his CSF and resolution of diffuse bone pain. Subsequent treatment with MTX-CPDG was noteworthy in that the pharmacokinetics of MTX decay from the CSF were altered with CPDG rescue, as compared to LV (12).

The fourth patient (case 7) had an adenocarcinoma of the breast metastatic to the CNS 5 years after a radical mastectomy and local radiation therapy for control of the primary tumor. The CNS metastases were initially treated with whole brain radiotherapy followed by cyclophosphamide, MTX, and 5-fluorouracil (CMF) with disappearance of the CNS lesions. Because of persistent neurological deficits, she received high-dose MTX (3 g/m²) with LV rescue for a total of 20 courses. Although neurologic symptoms resolved, new lesions developed. She then received three courses of MTX (10 g/m²) with CPDG rescue, which resulted in a 50% or greater decrease in the size of the CNS lesions by CT scan. Because of sensitization to CPDG, MTX-LV was begun again and progression of all lesions occurred. An Ommaya reservoir was then implanted and intraventricular C×T therapy was begun with MTX. The C×T courses were 3, 5, and then 7 days in length. After the third course, there was a substantial decrease in the size of one lesion and probable disappearance of another lesion. The patient has been off all therapy for 7+ months and currently has progressive CNS disease.

DISCUSSION

The administration of MTX can be manipulated by a variety of techniques to increase MTX levels in the CNS. At conventional oral doses, little if any MTX is found in CSF, and at single iv doses of 50 mg, peak levels of only slightly more than 10^{-8} M are detectable in ventricular CSF (16). In high-dose protocols, MTX overcomes blood-brain and blood-CSF barriers related to its relative lack of lipid solubility. MTX clearly crosses the blood-CSF barrier at doses of 500 mg/m² administered over 24 hours (16,17). Peak CSF MTX levels greater than 10^{-5} M have been detected in some high-dose MTX protocols (1,3). MTX has also been identified in autopsy specimens of brain tissue from patients treated with high-dose MTX (1). In those instances, MTX presumably also crossed the blood-brain barrier.

There are alternatives to LV for rescue after high-dose MTX administration (18–20). One of these, CPDG, has theoretical advantages over LV in that CPDG is a large molecule (21) and should be excluded from the CNS by the blood-brain barrier. In fact, CPDG activity was not found in CSF after iv administration (12). The pharmacokinetics of MTX egress from the CSF may also be more favorable after CPDG rescue due to a prolongation of the CSF t½ for MTX (12). Patients 2 and 7 clearly responded to MTX-CPDG treatment after disease progression during earlier treatment with MTX-LV. In both cases, however, the MTX dose was also increased from 3 to 10 g/m². The effects of altering the rescue from LV to CPDG cannot be separated from increasing the MTX dose. However, CSF MTX levels may persist for longer periods after CPDG than after LV (12).

The most direct way to achieve and maintain high MTX levels in the CNS is to administer MTX directly into ventricular CSF. Administration into the lumbar CSF is less desirable since ventricular fluid MTX levels vary considerably after such administration (16). C×T MTX administration for treatment of CNS leukemia has been shown to be effective and well tolerated when 1 mg of MTX is given every 12 hours. MTX levels in CSF were approximately 5×10^{-7} M in that study (6). After a 6 mg loading dose, we administered 2 mg of MTX every 12 hours. This resulted in CSF MTX levels in the range of 5×10^{-6} to 2×10^{-5} M. The patient with metastatic adenocarcinoma to the brain had a dramatic reduction in her CNS disease after her last 7-day C×T treatment. This response occurred following disease progression with both MTX-LV and MTX-CPDG.

MTX is an active agent for the treatment of certain CNS malignancies. Its efficacy has been established for both the prophylaxis and treatment of CNS involvement in acute lymphoblastic leukemia (8). Carcinomatous meningitis may also respond to MTX (22). In both circumstances, MTX is administered either intrathecally into the lumbar CSF or into the ventricular CSF via an Ommaya or Rickham reservoir. There is experimental (22) as well as clinical (6) evidence to suggest that frequent small intraventricular MTX doses that maintain CSF MTX levels are better tolerated and are possibly therapeutically superior to larger pulsed MTX doses (6). In addition, there are both theoretical (23) and experimental (24) data that suggest that MTX administered intraventricularly may equilibrate throughout extracellular fluid in the brain. The response seen in case 7 after C×T treatment further supports the contention that intraventricular MTX administration in a C×T fashion may diffuse through and into both the extracellular and intracellular brain compartments.

Our experience with CNS malignancies suggests that a variety of tumor types may respond to MTX through therapeutic approaches directed at maximizing the CSF MTX concentration and the time of drug exposure.

REFERENCES

1. Rosen G, Ghavimi F, Nirenberg A, et al. High-dose methotrexate with citrovorum factor rescue for the treatment of central nervous system tumors in children. Cancer Treat Rep 61:681–690, 1977.
2. Djerassi I, Sun Kim J, and Shulman K. High-dose methotrexate-citrovorum factor rescue in the management of brain tumors. Cancer Treat Rep 61:691–694, 1977.
3. Pitman SW, and Frei E (iii). Weekly methotrexate-calcium leucovorin rescue: effect of alkalinization on nephrotoxicity-pharmacokinetics in the CNS; and use in CNS non-Hodgkin's lymphoma. Cancer Treat Rep 61:695–701, 1977.
4. Shapiro WR. High-dose methotrexate in malignant gliomas. Cancer Treat Rep 61:753–756, 1977.
5. Skarin AT, Zuckerman KS, Pitman SW, et al. High-dose methotrexate with folinic acid in the treatment of advanced non-Hodgkin lymphoma including CNS involvement. Blood 50:1039–1047, 1977.
6. Bleyer WA, Poplack DG, Simon RM, et al. "Concentration × time" methotrexate via a subcutaneous reservoir: a less toxic regimen for intraventicular chemotherapy of central nervous system neoplasms. Blood 51:835–842, 1978.
7. Shapiro WR, Posner JB, Ushio Y, et al. Treatment of meningeal neoplasms. Cancer Treat Rep 61:733–743, 1977.
8. Simone JV, Hustu HO, and Aur RJA. Prevention and treatment of central nervous system leukaemia in children. *In* CNS Complications of Malignant Disease (Whitehouse JMA, and Kay HEM, eds). London, MacMillan Press, Ltd., 1979.
9. Wilson CB. Brain tumor chemotherapy with intrathecal methotrexate. Cancer 23:1038–1045, 1969.
10. Newton WA, Sayers MP, and Samuels LP. Intrathecal methotrexate therapy for brain tumors in children. Cancer Treat Rep 52:257–261, 1968.
11. Ratcheson RA, and Ommaya AK. Experience with the subcutaneous cerebrospinal fluid reservoir—preliminary report of 60 cases. N Engl J Med 279:1025–1031, 1968.
12. Abelson HT, Ensminger W, Rosowsky A, et al. Comparative effects of citrovorum factor and carboxypeptidase G_1 on cerebrospinal fluid-methotrexate pharmacokinetics. Cancer Treat Rep 62:1549–1552, 1978.
13. Raso V, and Schreiber R. A rapid and specific radioimmunoassay for methotrexate. Cancer Res 35:1407–1410, 1975.
14. Abelson HT, Ensminger W, Kufe D, et al. High-dose methotrexate-carboxypeptidase G_1—a selective approach to the therapy of central nervous system tumors. *In* Chemistry and Biology of Pteridines (Kisliuk RL, and Brown GM, eds). New York, NY, Elsevier, 1979, pp 629–633.
15. Canellos GP, Skarin AT, Ervin T, et al. A chemotherapeutic approach to CNS lymphoma and leukaemia by the systemic administration of high doses of antimetabclites. *In* CNS Complications of Malignant Disease (Whitehouse JMA, and Kay HEM, eds). New York, NY, MacMillan, 1979, pp 142–148.
16. Shapiro WR, Young DF, and Mehta BM. Methotrexate: distribution in cerebrospinal fluid after intravenous, ventricular, and lumbar injections. N Engl J Med 293:161–166, 1975.
17. Freeman AI, Wang JJ, and Sinks LF. High-dose methotrexate in acute lymphocytic leukemia. Cancer Treat Rep 62:727–731, 1977.
18. Ensminger WD, and Frei E (iii). The prevention of methotrexate toxicity by thymidine infusions in humans. Cancer Res 37:1857–1863, 1977.
19. Howell SB, Ensminger WD, Krishan A, et al. Thymidine rescue of high-dose methotrexate in humans. Cancer Res 38:325–330, 1978.
20. Bertino JR. Toward improved selectivity in cancer chemotherapy. Cancer Res 39:293–304, 1979.
21. McCullough JL, Chabner BA, and Bertino JR. Purification and properties of carboxypeptidase G_1. J Biol Chem 246:7207–7213, 1971.
22. Shapiro WR, Posner JB, Ushio Y, et al. Treatment of meningeal neoplasms. Cancer Treat Rep 61:733–743, 1977.
23. Blasberg RG, Patlak CS, and Shapiro WR. Distribution of methotrexate in the cerebrospinal fluid and brain after intraventricular administration. Cancer Treat Rep 62:633–641, 1977.
24. Kimelberg HK, Kung D, Watson RE, et al. Direct administration of methotrexate into the central nervous system of primates. Part 1: distribution and degradation of methotrexate in nervous and systemic tissue after intraventricular injection. J Neurosurg 48:883–894, 1978.

High-Dose Oral Methotrexate

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SUMMARY

In this study nine patients were treated with 250 mg of methotrexate (MTX) in divided oral doses every 2 weeks. Pharmacokinetic studies carried out on alternate courses showed no alteration with cycles in peak levels of the drug, the half-life of MTX, or the area under the concentration-time curve. Two partial responses (PR) were noted in head and neck squamous carcinoma. Three patients had stable disease and four progressed. Oral high-dose MTX produced predictable exposure of tumors to MTX, although the bioavailability was always less than the equivalent iv dose. Oral MTX was well tolerated by patients, toxicity was minimal, and no evidence of malabsorption of the drug was detected.

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Despite the controversies surrounding the pharmacology of methotrexate (MTX), there seems general agreement that over a dose range between 30 mg and 100 mg the drug is more reliably available when given iv than orally. Our previous work with the dose of 50 mg/m² given to six patients by each route, 2 weeks apart, showed that the highest percentage bioavailability achieved by the oral route was 50% of the equivalent dose given iv and the lowest was 20% (1).

We have shown, however, that the amount of MTX that potentially might enter tumor cells can be increased by subdividing an oral dose (2). For the oral route to have any value in the outpatient treatment of cancer patients, maximal absorption must be achieved. There is little doubt that patients prefer taking a drug orally to receiving it by injection, and that it is cheaper to treat outpatients than inpatients. There are two potential problems in raising the dose of oral MTX above 50 mg. The first is that relatively less MTX may be absorbed as the dose is increased, assuming that at least part of the drug will be transported across the gut mucosa by an active system. This might be avoided by divided dosages. The second problem is that MTX may damage the gut and thereby jeopardize the absorption of subsequent doses. There is evidence from Craft et al (3) that children given chronic low-dose MTX may suffer a degree of malabsorption of xylose. We have previously established by a Latin square study that absorption is linear over the dose range 25 mg to 100 mg, although the rate of absorption varies widely from patient to patient. A dose of 50 mg seemed reliably absorbed from these studies and so a regime applying 50 mg MTX every 2 hours to a total of 250 mg (ie, five doses) followed by folinic acid (CF) rescue at 24 hours was tested in patients with squamous cancers of cervix, penis, and head and neck in order to answer the above two questions.

METHODS

Nine patients with histologically proven measurable squamous carcinoma were selected for study. Each had adequate renal, liver, and marrow function, a performance of 2 or better (ECOG), and had not received any prior chemotherapy. Each patient received 250 mg of MTX by mouth every 2 weeks for eight courses. Five 10 mg tables of MTX were administered every 2 hours for five doses and this was repeated every 2 weeks. CF rescue consisted of 15 mg CF qid by mouth for 2 days following 1 day after commencement of MTX. The pharmacokinetic analysis of MTX was carried out on the first, third, fifth, and seventh cycles of MTX. Pretreatment plasma and urine samples were obtained and, thereafter, 19 appropriately timed plasma samples were drawn from an indwelling iv plastic catheter flushed with heparin. Urine collections were obtained every 6 hours for 72 hours. MTX was measured by means of an enzyme immunoassay method using the EMIT® system and random samples were checked by ra-

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dioimmunoassay (1) for reproducibility. Complete results are available for eight of the nine patients, although one patient received his final dose of MTX in a 12-hour infusion. The ninth patient has not yet reached the seventh cycle, but the results of the first three samplings are presented.

RESULTS

The drug was well tolerated by all patients and no serious toxicity was seen. A total of 70 cycles of MTX was available for analysis of toxicity. Only one patient experienced toxicity sufficient to delay a subsequent cycle for 1 week (wbc less than 3,000/mm^3 or platelet count less than 100,000/mm^3). Two partial responses were seen in patients with head and neck cancers. Disease stabilization was seen in three patients. Four patients had evidence of disease progression on the regimen and were then treated with 2 g/m^2 of MTX by infusion. No responses were seen in these four patients. Table 1 shows the results of the pharmacokinetic evaluation in nine patients. The peak levels of MTX varied between 1.34 and 5.34 μM, although the emphasis on the peak level is probably not particularly relevant because of the subdivision of the dose of MTX. In most patients an accumulation was not reflected in the peak level, rather three of four peaks of the same order of magnitude were observed. There was no significant change in the β half-life or the area under the concentration-time curve throughout the seven cycles of study. We thus concluded that malabsorption was not induced by these pulses of MTX or was reversed between cycles. No change in kinetics of MTX was observed throughout the intermittent protocol. Excellent correlation between the two assay systems was observed in 60 random samples, though the EMIT® method recorded 30% higher than the radioimmunoassay at all concentrations.

TABLE 1.—Areas under curves and β half-lives ($t_{1/2}$) after oral MTX

Patient		Course 1	Course 3	Course 5	Course 7
1	$t_{1/2}$	1.67	1.82	1.75	1.58
	AUC	13485	21190	20562	22765
2	$t_{1/2}$	2.05	1.85	1.98	2.10
	AUC	12654	11421	13610	13982
3	$t_{1/2}$	2.50	2.00	2.42	2.35
	AUC	18228	17903	15588	12389
4	$t_{1/2}$	1.82	1.75	1.94	2.03*
	AUC	20430	18293	19762	28476
5	$t_{1/2}$	2.18	2.42	2.00	1.98
	AUC	15625	14876	15982	16015
6	$t_{1/2}$	1.72	1.59	1.64	1.78
	AUC	19572	18521	20147	19872
7	$t_{1/2}$	1.95	1.82	1.94	2.04
	AUC	14162	15252	14928	16572
8	$t_{1/2}$	1.60	1.58	1.50	1.72
	AUC	16262	18451	17561	15742
9	$t_{1/2}$	1.58	1.64	1.53	-
	AUC	20057	20921	19765	-

*iv infusion ($t_{1/2}$=h, AUC=ng h ml^{-1})

DISCUSSION

The absorption of a drug depends on many variables; therefore, the potential for interference with absorption either by the introduction of other drugs or by impedance with gastric emptying implies that the use of this route will always be slightly precarious. On the other hand, the present figures show that for a specific dose of drug given every 2 weeks, the bioavailability of MTX is standard and predictable for an individual patient. No malabsorption ensued over at least seven courses of treatment. No alteration in renal clearance of MTX occurred either. Patients preferred this schedule to iv infusions. Antitumor responses, although minor, did occur. Oral MTX might now be incorporated into a combination protocol with some confidence that a certain degree of drug absorption will occur.

We have studied several patients' MTX levels after an infusion of an identical total dose of MTX (250 mg). The range of bioavailability is equivalent to that shown for patient No. 4, who had his fourth study carried out on an iv dose. Without exception the areas under the C-T curves at this dose and at every lesser dose we have studied are significantly higher with iv administration than those achieved by the equivalent dose administered by mouth. Multiple reasons for this may exist; it may be related to first pass metabolism of MTX in the gut or may be caused by flooding of the supposed active transport mechanism by which the enterocyte handles MTX. A theoretical solution to this problem might be the administration of MTX into the rectum. An analogous situation exists with several β-blocking agents; bioavailability from rectal administration of these drugs has proved to be equal to that from the iv route and is successful in overcoming the obstacle of first pass metabolism. This study did not include tests of absorption of substances other than MTX, such as xylose, glucose, or folate. Biochemical parameters, however, including liver function tests and serum proteins, did not alter the course of the study of these nine patients. So, if malabsorption was taking place, it was to a small degree and of questionable relevance to the patient. Other workers have shown that doses up to 800 mg given in divided schedules by mouth may be adequately absorbed and equivalent to the iv route (4).

Christophidis and coworkers have treated 14 patients with MTX in combination with three other drugs (cyclophosphamide, Adriamycin, and vincristine). Patients were studied on one occasion. Their figures for oral absorption are consistent with those reported in our study, but their corresponding iv route figures are considerably lower than would be predicted from data we have collected from a number of patients given iv doses up to 2 g/m^2. Since half of the patients were given chemotherapy in an adjuvant protocol, tumor responses cannot be assessed. Evans and associates (5) have reported pharmacokinetic data using doses in the same range as the present study. They reported areas under the curve for a 4-

hour study compared to 72 hours in the present communication, and showed escalation with increasing dose as we have done. The peak levels were also in agreement. Again, this report merely pertains to a single study on each patient and the number of patients was very small. No response data were given.

CONCLUSION

Although the bioavailability of MTX given by the oral route was less than when given iv, the regimen resulted in exposure of the tumor to predictable levels of the drug and was well tolerated. Furthermore, two patients responded to the regimen and three patients had stabilization of their disease.

REFERENCES

1. Stuart JFB, Calman KC, Watters J, et al. Bioavailability of methotrexate: implications for clinical use. Cancer Chemother Pharmacol. In press.
2. Steele WH, Stuart JFB, Lawrence JR, et al. Enhancement of methotrexate absorption by subdivision of dose. Cancer Chemother Pharmacol. In press.
3. Craft AW, Kay HEM, Lawson DN, et al. Methotrexate-induced malabsorption in children with acute lymphoblastic leukaemia. Br Med J 2:1511–1512, 1977.
4. Christophidis N, Vajda FJE, Lucas I, et al. Comparison of intravenous and oral high-dose methotrexate in treatment of solid tumours. Br Med J 1:298–300, 1979.
5. Evans W, Rivera G. Pharmacokinetic evaluation of high-dose oral methotrexate (MTX) in children with acute lymphocytic leukemia (ALL). Proc Am Assoc Cancer Res 20:266, 1979.

Treatment of Advanced Head and Neck Carcinoma with Synchronous Irradiation and Methotrexate

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SUMMARY

Thirty-four patients with advanced head and neck cancer were treated by synchronous radiotherapy and methotrexate (MTX) (100 mg/m²). Complete resolution of disease was obtained in 18 patients, the median remission of patients being 17 months. In general, treatment was well tolerated, although mucosal reaction was prolonged in 50% of patients. Drug-induced hematological toxicity was observed in six patients (18%) and one patient died. As a result of these findings, a random clinical trial to compare X-ray therapy with X-ray therapy and MTX in advanced head and neck cancer has been commenced.

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There has been continued interest during the last two decades in the combination of radiotherapy and chemotherapy for the management of head and neck cancer. The majority of T1 and T2 lesions without nodal involvement are cured by either radiotherapy or surgery, but the prognosis for patients with advanced lesions, particularly those with nodal disease on presentation, is poor; up to 90% may be dead within a year of diagnosis (1). Various cytotoxic drugs or drug combinations have been used in conjunction with radiotherapy in an attempt to improve survival, but with conflicting results. This applies particularly to methotrexate (MTX), and may be explained in part by the complex pharmacology of the drug (2,3). The ability now to detect potentially toxic serum concentrations and thereby prevent serious toxicity has permitted the reexamination of the efficacy of moderate dose MTX without rescue. In view of this, and because there is some experimental evidence that MTX may act as a radiosensitizer (4), we elected to undertake a pilot study of radiation and synchronous dose MTX in patients with advanced carcinoma of the head and neck.

PATIENTS, MATERIALS, AND METHODS

Patients

Only patients with histologically proven advanced carcinoma of the head and neck, judged to be beyond cure with surgery or conventional radiotherapy, were included in the study. All patients were staged by the TNM classification of the International Union Against Cancer (UICC). No age limit was placed on selection and poor general condition did not preclude therapy. The following investigations were performed in all patients prior to treatment: hematological and biochemistry profile (SMA 12), chest X-ray, serum and red cell folate, urea, electrolytes, and creatinine clearance. Bone marrow aspiration and trephine were performed only if indicated by hematological screening tests.

Treatment

Radiotherapy was delivered by a 4 MV accelerator using standard beam direction techniques to ensure dose homogeneity. In all cases the tolerance dose of radical radiotherapy was prescribed based on experience with MV photon therapy over many years. Treatment was planned and delivered in a standard fractionation regime of 16 fractions in 21 days. The absolute doses delivered ranged from 3,000 rad (cGy) to 5,500 rad (cGy), but the majority of patients received doses in the range 4,500 to 5,500 rad. This established radical technique produces a fibrinous mucosal reaction at 10 days that lasts 4 to 6 weeks

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after completion of radiation therapy. Skin reactions beyond dry desquamation are rarely seen.

MTX (100 mg/m²) was administered as a single iv push 24 hours prior to commencement of radiotherapy and 14 days later. The first nine patients received one dose so that information concerning toxicity could be collated. When it became apparent that this was within tolerance, a second dose was administered in a like manner on Day 14. Twenty-four hours after each dose a 5 ml sample of blood and two consecutive 12-hour urine collections were obtained for determination of serum concentration and cumulative 24-hour excretion respectively. Drug concentrations were determined by radioimmunoassay using a modification of the method of Aherne (5). Leucovorin (15 mg every 6 hours × 4 doses po) was administered if the 24-hour concentration was > 0.6 μm.

Toxicity

Hematological toxicity was assessed by biweekly full blood counts. Mucosal reaction was assessed *by means of a rating scale.* Other toxicities were recorded *as they arose.* After completion of therapy, patients were followed at monthly intervals for 6 months and then at increasing intervals thereafter. Successful therapy was defined as complete resolution of disease without added toxicity.

RESULTS

Thirty-four patients (mean age 59 years), 23 male and 11 female, received treatment during the period January 1978 to July 1978. Details of site of disease and mean age are given in table 1 and clinical stage is given in table 2. Most patients had advanced disease, 27 (80%) presenting with stage IV and the remainder with stage III disease. The dose of radiotherapy ranged from 3,000 to 5,500 rad, according to the site and extent of tumor. Five patients had evidence of folate depletion prior to therapy and six had a creatinine clearance of > 50 ml/minute.

Nine patients received one dose of MTX (MTX 1); seven received 100 mg/m², but the dose was reduced in two due to poor general condition and pretreatment folate status. A second dose (MTX 2) was administered to 25 patients, of whom 18 received 100 mg/m², while this was reduced in seven patients because of folate depletion or impaired creatinine clearance. The mean 24-hour serum concentration following MTX 1 was 0.22 μm (range, 0.05 to 1.3) for MTX 2 was 0.23 μm (range, 0.04 to 0.85). Two patients required leucovorin rescue.

One patient died as a result of drug toxicity; therefore, 33 patients were assessable for response. Complete resolution was obtained in 18 patients (53%). Two of these subsequently died from incidental causes, one in a road traffic accident at 8 months, and one from second primary that developed 15 months after successful therapy. The remaining patients are alive and disease free with a median remission duration of 17 months (range 12 to 21). Seven patients are alive with disease (median survival 13 months, range 12 to 20 months) and block neck dissection was performed on two of these patients. Ten patients have died, all with advanced disease, and eight within 10 months of completing therapy. Further chemotherapy with high-dose MTX (HDMTX) was attempted in two patients with no response. Seven patients had very advanced carcinoma of the oral cavity and would not normally have been considered for therapy.

The survival of the 33 patients is shown in figure 1. The overall survival at 12 months was 69%, falling to 63% at 21 months.

TABLE 1.—Sites of disease and mean (+ SD) of 34 patients with advanced head and neck carcinoma treated with synchronous MTX and DXR

Site	N	Age
Oral cavity	7	64 ± 4.2
Oropharynx	8	57 ± 4.6
Nasopharynx	1	55
Hypopharynx	10	60 ± 9
Larynx	5	58 ± 4
Antrum	2	65 ± 1
Unknown primary	1	19
Total	34	59 ± 9

TABLE 2.—Clinical stage (TNM) of 34 patients with advanced head and neck cancer [1]

	T2	T3	T4
N0	-	4	2
N1	2	4	0
N2	1	3	0
N3	6	10	1

[1] Excludes one patient with squamous carcinoma of cervical lymph nodes from unknown primary.

Toxicity

In general, treatment was well tolerated. The radiotherapeutic technique employed at this Institute inevitably produced a fibrinous mucosal reaction that appears about the 10th to 12th day. It was therefore impossible to determine the contribution of MTX 1 to this phenomenon. The reaction was, however, confined to the irradiated volume. In only three patients was the mucosal reaction considered to be more severe than the norm, although the reaction started 2 to 3 days earlier. In approximately 50% of patients healing was delayed, the normal reaction persisting 4 months compared to the normal 2 months. No other morbidity occurred. During therapy no patients required iv fluids or nasogastric feeding, nor was it

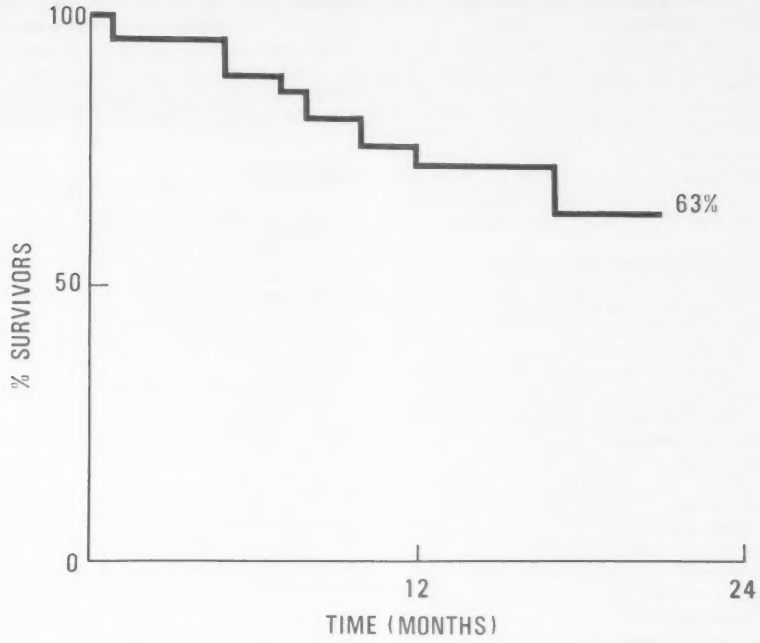

FIGURE 1.—Survival of 33 patients with advanced squamous carcinoma of the head and neck.

necessary to reduce the planned radiotherapeutic dose for any patient.

Hematological toxicity to MTX is shown in table 3. Two patients, one of whom died, developed neutropenia. Two patients developed a skin rash, and one patient developed renal toxicity. No episode of respiratory tract infection was observed. One episode of septicemia, which was successfully treated with appropriate antibiotic therapy, developed in a patient with neutropenia.

Table 3.—Hematological toxicity in 34 patients with advanced head and neck carcinoma treated with synchronous MTX and DXR

Parameter	MTX 1 (n=34)	MTX 2 (n=25)
wbc $< 3.0 \times 10^9/l$	6 (18%)	3 (12%)
Neutrophils $< 1.0 \times 10^9/l$	4 (12%)	3 (12%)
Platelets $< 80 \times 10^9/l$	4 (12%)	0 (–)

DISCUSSION

The treatment of advanced head and neck carcinoma is unsatisfactory. The combination of chemotherapy with radiation is one option to attempt to improve survival.

MTX is the most effective single agent in treating head and neck carcinoma and has been given in combination with radiotherapy with conflicting results. Condit et al administered various doses by different routes but could draw no definitive conclusions (6). Kligermann administered MTX 15 days prior to irradiation with possible benefit to those patients who responded to the drug (7). Kramer used MTX before and during irradiation with possible beneficial effects (8) and Lustig concluded that MTX given continuously until stomatitis developed significantly improved survival (9). Their observations contrast with those of Knowlton et al, who could detect no significant difference in survival at 3 to 5 years with both low- and high-dose MTX followed by irradiation (10).

Drug combinations have also been used synchronously with radiation; O'Connor et al, using a combination of vinblastine, MTX, and bleomycin, were impressed by the apparent increase in survival (61% at 2 years and 52% at 30 months) (11,12). However, one-third of the patients required nasogastric feeding, the dose of radiation in several patients had to be interrupted because of severe stomatitis, and five fatalities occurred from infective complications. Fu et al, using a combination of cyclophosphamide, vincristine, and bleomycin during irradiation, observed 3 drug deaths

in a group of 15 and concluded that this particular combination was impractical (13).

It is probable that the optimum administration of MTX has yet to be defined. In all the above studies, with one exception, the drug was administered *before* radiation, presumably to minimize the development of stomatitis. It is possible, however, that the timing of the dose and drug may be crucial if improved results are to be achieved. For this reason, therefore, this pilot study was initiated to explore the combination of radiation and MTX when the drug is given concurrently.

Although MTX has been widely used for 3 decades, there is still conflict over the optimum dose. The dose selected here, 100 mg/m², was chosen following a detailed kinetic study in patients with head and neck disease. With this dose, a concentration of 1 μm is maintained for at least 12 hours, and this concentration is in excess of that considered to be necessary for cytotoxic effect (14), whereas a concentration of 0.01 μm will produce hematological toxicity but has no cytotoxic effects (15,16). This dose can and will produce severe toxicity, but reliability in monitoring drug concentrations at 24 hours permits rescue of those patients with potentially toxic concentrations. The one fatality that occurred in this series was in a patient in poor general condition with folate depletion. In addition, a retrospective serum analysis revealed that no biotransformation had occurred in this patient, whereas in the remaining patients, substantial amounts of 7-hydroxy-MTX were produced at 24 hours.

The prime aim of this study was to determine whether it would be possible to administer a cytotoxic drug in conjunction with an effective biological dose of radiation. It seemed to us that the prime requisites of synchronous treatment are that the fractionated dose must not be interrupted because of drug toxicity and that whichever cytotoxic drug is chosen should be given in optimum dose in conjunction with radiation. This is obligatory if optimum effect is to be achieved. These objectives have been met and, although some patients experienced an exaggerated mucosal reaction, treatment was generally well tolerated and no patient required artificial feeding. In some patients there was excellent resolution of both the primary and nodal disease, which was reflected in the survival of patients who were considered initially to be beyond curative treatment with radiation alone. The survival rate obtained in this study is similar to that of Clifford et al (12). The particular drug combination used in the Clifford study produced more toxic effects and, in many patients, interruption of the planned radiotherapeutic dose was necessary.

In view of these results, we have now proceeded to a prospective randomized control trial in patients with less advanced disease to determine whether these observations will be substantiated.

REFERENCES

1. Stell PM. The management of cervical lymph nodes in head and neck cancer. Proc Roy Soc Med 68:83–85, 1974.
2. Bleyer VA. Methotrexate: clinical pharmacology, current status and therapeutic guide lines. Cancer Treat Rev 4:87, 1977.
3. Wilkinson PM, Morice ER, and Lucas SB. Clinical and kinetic parameters that influence the response and toxicity to methotrexate. *In* Clinical Pharmacology of Antineoplastic Drugs (Pinedo HM, ed). Elsevier/North Holland Biomedical Press, 1978, pp 29–38.
4. Berry RJ, and Asquith JS. Cell cycle-dependent and hypoxic radiosensitizers. *In* Advances in Chemical Radiosensitization. Vienna, International Atomic Energy Agency, 1974, pp 25–36.
5. Aherne GW, Piall EM, and Marks V. Development and application of a radioimmunoassay for methotrexate. Br J Cancer 36:608–617, 1977.
6. Condit PT, Ridings GR, Coin JW, et al. Methotrexate and radiation in the treatment of patients with cancer. Cancer Res 24:1524–1533, 1964.
7. Kligermann MM, Hellman S, Von Essen CF, et al. Sequential chemotherapy and radiotherapy. Radiology 86:247–250, 1966.
8. Kramer S. Use of methotrexate and radiation therapy for advanced cancer of the head and neck. *In* Frontiers of Radiation Therapy and Oncology, vol. 4. Karger, Basel, 1969, pp 116–125.
9. Lustig RA. Adjuvant methotrexate in the radiotherapeutic management of advanced tumours of the head and neck. Cancer 37:2703–2708, 1976.
10. Knowlton AH, Percarpio B, Borrow S, et al. Methotrexate and radiation therapy in the treatment of advanced head and neck tumours. Radiology 116:709–716, 1975.
11. O'Connor AD, Clifford P, Durden-Smith DJ, et al. Synchronous VBM and radiotherapy in the treatment of squamous cell carcinoma of the head and neck. Clin Otolaryngol 1:347–357, 1977.
12. Clifford P, O'Connor AD, Durden-Smith J, et al. Synchronous multiple drug chemotherapy and radiotherapy for advanced (stage III and IV) squamous carcinoma of the head and neck. Antibiot Chemother 24:60–72, 1978.
13. Fu KK, Silverberg IJ, Phillips TL, et al. Combined radiotherapy and multi-drug chemotherapy for advanced head and neck cancer. Results of a radiation therapy oncology group pilot study. Cancer Treat Rep 63:351–357, 1979.
14. Calvertah AH, Bondy PK, and Harrap KR. Some observations on the human pharmacology of methotrexate. Cancer Treat Rep 61:1647–1656, 1977.
15. Hryniuk WM, and Bertino JR. Treatment of leukaemia with large doses of methotrexate and folinic acid: clinical biochemical correlates. J Clin Invest 48:2140–2155, 1969.
16. Pinedo HM, Zaharko DS, Bull J, et al. The relative contribution of drug concentration and duration of exposure to mouse bone marrow toxicity during continuous methotrexate infusion. Cancer Res 37:445–450, 1977.

Safe and Effective Combination Chemotherapy Without Cis-Platinum for Squamous Cell Carcinomas of the Head and Neck [1]

L. A. Price [2]* and Bridget T. Hill [3]

SUMMARY

Different combinations of "standard" antitumor drugs were given to patients with squamous cell carcinomas of the head and neck. One hundred fifty-five patients received a combination of vincristine, bleomycin, methotrexate, hydrocortisone, and 5-fluorouracil given over 24 hours every 2 to 3 weeks. In patients who had received no previous treatment, 57 of 76 assessable patients (75%) responded. In previously treated patients, 20 of 40 assessable patients responded (50%). Prior radiation significantly reduced response to chemotherapy. A response to initial chemotherapy increases the likelihood of successful local treatment. In this study, response to drug treatment was not affected by age, sex, histology, or site. Crude survival at 2 years is significantly increased in responding patients compared with a historically matched control group when chemotherapy is given before radiation therapy or surgery. Preliminary results of a randomized prospective controlled study suggest that primary adjuvant chemotherapy without cis-platinum may significantly increase survival in this group of patients.

[Cancer Treat Rep 65 (Suppl. 1):149–154, 1981]

In 1969 we first demonstrated that very high doses (eg, 20 g) of methotrexate (MTX) could be given safely and also produce further responses in patients resistant to lower, more orthodox doses (1,2). The duration of these responses, however, was relatively short, and so the high-dose MTX program was abandoned in the early 1970's in favor of a series of studies using combinations of antitumor drugs. Initially, a combination using a number of drugs with single agent activity was used in the hope that it would show a significant effect. The plan, which was to simplify the protocol, subsequently led to the following questions:

1. Is it possible, using a kinetically based approach, to design an effective combination of standard agents for this group of tumors?

2. Using the same theoretical basis, is it possible to simplify the protocol without loss of therapeutic effect and with minimal toxicity?

3. If a safe and effective protocol can be devised, does its addition to standard local therapy with radiation and/or surgery increase survival?

A pilot study to answer the first question was carried out in 1974. Thirty-six patients with advanced, previously treated squamous cell carcinomas of the head and neck were treated with a seven-drug combination, scheduled as shown in figure 1 and repeated every 3 weeks. Of 24 assessable patients, 19 showed greater than 50% tumor regression, and complete remissions were seen in 3. No significant toxicity occurred. Although this study proved that very high response rates could be achieved safely in patients with advanced tumors (3), the overall survival rate again was not impressive, with a median duration of survival of approximately 4 to 5 months (fig 2). Accordingly, when the protocol was simplified in order to answer question 2, some patients were given chemotherapy as initial treatment.

The protocol used in our second study is shown in figure 3. The hydrocortisone was included in an attempt to block the immediate and long-term side effects of bleomycin; otherwise the same kinetic basis was used as in the 1974 protocol. This underlying theoretical basis has been detailed elsewhere (4). One hundred fifty-five patients received this protocol, and the following definitions were used during the study:

Group I: New patients receiving chemotherapy *before* planned radiation or surgery. These patients

[1] We are particularly indebted to Dr. Hilary Calvert for the statistical analysis of these data and to Mr. Henry Shaw and Dr. Vera Dalley for referring patients into this study. We would also like to thank Mrs. Joan Isaac for expert secretarial assistance.

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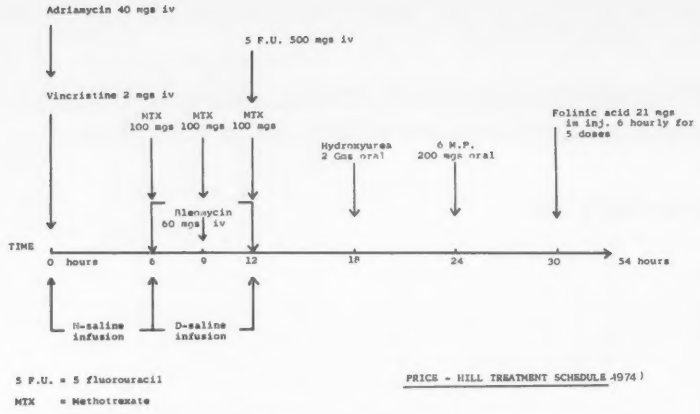

FIGURE 1.—The initial seven-drug protocol used during 1974.

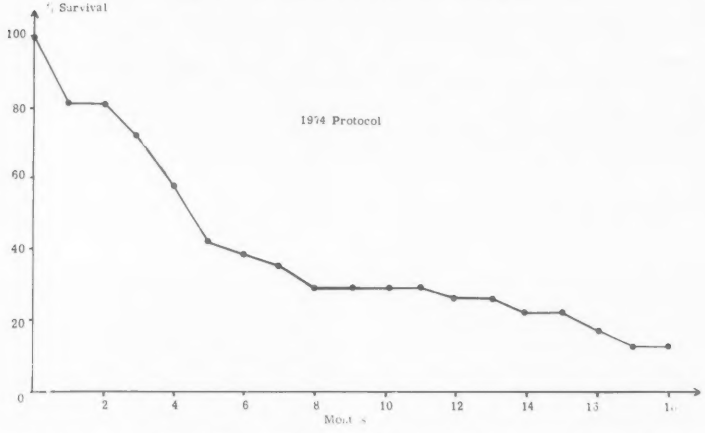

FIGURE 2.—Actuarial survival of patients treated on the 1974 protocol. All patients, including those not assessable for response, were included in this analysis.

were given chemotherapy on days 1 and 14 and started on local therapy (radiation or surgery) on day 28.

Group II: Patients with recurrent, previously treated disease, or metastases, who were considered untreatable except by chemotherapy. These patients were given chemotherapy every 3 weeks, until maximal response or disease progression occurred.

Response: A reduction of at least 50% in the product of two perpendicular diameters of all measurable lesions.

Price Hill Schedule A

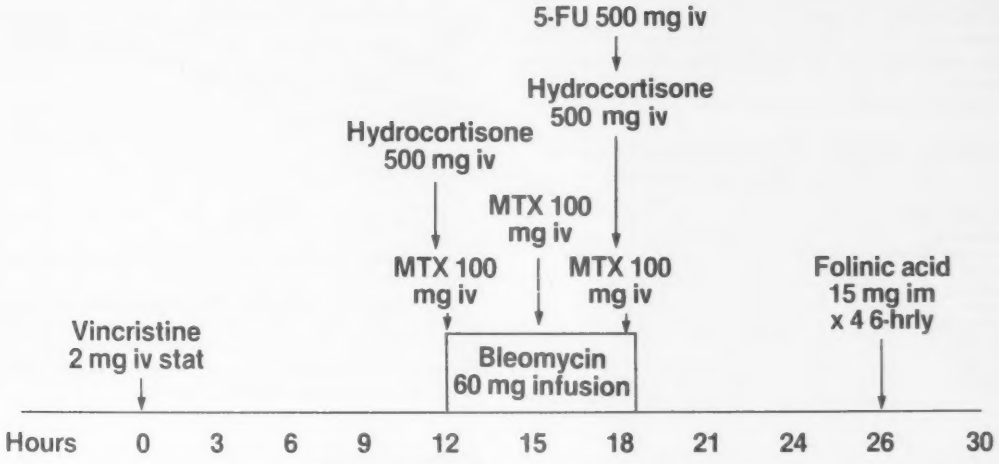
5-FU 500 mg iv
Hydrocortisone 500 mg iv
Hydrocortisone 500 mg iv
MTX 100 mg iv
MTX 100 mg iv
MTX 100 mg iv
Folinic acid 15 mg im x 4 6-hrly
Vincristine 2 mg iv stat
Bleomycin 60 mg infusion
Hours 0 3 6 9 12 15 18 21 24 26 30

FIGURE 3.—The simplified four-drug protocol used since 1975.

Table 1 summarizes the characteristics of the patients in Group I.

TABLE 1.—Characteristics of Group I patients receiving initial chemotherapy

Mean age 56. Total number of courses 203: TNM staging (ALL M0)

	N0	N1	N2	N3
T1	6	5	1	1
T2	18	10	3	4
T3	23	12	3	4
T4	2	5	2	1

STATISTICAL METHODS

Response rates were compared using the chi-squared test with Yates' correction. Survival was calculated by a life table method and compared using the log rank test (5).

RESULTS

Group I

Initial Schedule A chemotherapy was given to 101 ambulant patients, of whom 76 were assessable. Reasons for nonassessability were: inadequate measurement, 14; alternative treatment started too soon, 5; inadequate followup, 3; died before 50% reduction, 3. Fifty-seven patients responded (75%). Side effects, which were minimal, are summarized in table 2.

TABLE 2.—Group I—Side effects

Myelosuppression	1 (died, protocol violation)*
Neuropathy	4 (mild paraesthesia)
Pulmonary fibrosis	0
Alopecia	1
Atrial fibrillation	1
Malaise	8
Anorexia and nausea	8
Skin rash	4
Mucositis	4 (no intubation)
Abdominal pain	1
Ankle edema	1
Constipation	3

*This patient had impaired renal function but was not given an extended folinic acid "rescue."
No patients needed nasogastric tube feeding.

Group II

Schedule A chemotherapy was given to 54 previously treated patients; 6 patients needed help with walking but the others were ambulatory. Forty patients were assessable for response. Reasons for nonassessability were: inadequately measured lesions, 4; died within 2 weeks, 2; concurrent radiation given, 3; inadequate records, 5. Twenty patients responded (50%). This response rate is significantly lower than that observed in Group I ($P < 0.05$). Side effects in Group II patients are summarized in table 3.

TABLE 3.—Group II—Side effects in patients given chemotherapy after local treatment

Myelosuppression	0
Neuropathy	0
Pulmonary fibrosis	2
Alopecia	2
Cardiovascular	2
Malaise	6
Anorexia and nausea	5
Skin rash	2
Confusion	2
Mucositis	1 (no intubation)

SURVIVAL

These results are summarized in table 4 and in figures 4 and 5. In this analysis, in contrast to our earlier data (6), one patient previously classified as nonassessable has been reevaluated as a nonresponder and has survived more than 2 years. The overall survival of patients in Group I compared favorably ($P < 0.05$) with the survival of a historical control group of patients treated at the same hospital prior to the use of chemotherapy (data not shown). The survival of responders in Group I was better than that of nonresponders, although at the time of writing the difference was not statistically significant ($P = 0.14$). In this group, 78% of patients overall achieved a complete remission following radiation and/or surgery. Patients who responded to initial chemotherapy achieved an 81% complete remission rate after local treatment. However, only 63% of the patients who failed to respond to chemotherapy achieved a complete remission following local therapy. This difference is statistically significant ($P = 0.014$). Therefore a response to initial chemotherapy increases the likelihood of successful local treatment. Only 12 patients who did not respond to chemotherapy in Group I achieved a complete response following local treatment. Of these, 33% were alive at 2 years. This figure is less than that for chemotherapy responders, but the number of patients in this group is too small to draw any statistically valid conclusions.

TABLE 4.—Overall response rates and crude survival at 2 years

Group I:		
76 assessable patients.	50%	Response rate 75%
Historical control group.	25%	
Group II:		
40 assessable patients.	0%	Response rate 50%
Median duration of survival:		
Responders	320 days	$P = .06$
Nonresponders	150 days	

Note reduction in response rate and survival in previously irradiated patients.

In Group II, patients who responded to chemotherapy also survived longer than nonresponders, with a 170-day increase in the median survival time. However, this increase was of limited clinical benefit, since no patient survived to 2 years.

CONCLUSIONS

In previously nonirradiated patients:

1. Price-Hill Schedule A has a consistently high response rate that compares favorably with any other current protocol and is less toxic than most.
2. The automatic inclusion of cis-platinum in initial chemotherapy protocols remains to be justified.
3. A response to initial Schedule A chemotherapy increases the likelihood of successful local treatment.
4. Initial chemotherapy with this protocol significantly prolongs survival compared with a historical control group, and a randomized prospective controlled study is under way. The prospects for an eventual increase in survival in this trial are encouraging, since (a) a similar protocol given concurrently with radiation appears to have improved the prognosis (7), and (b) the use of our 1974/1975 protocol, which has a similar response rate, in a randomized controlled study shows increased survival when compared with local therapy alone (8).

In previously irradiated patients:

1. Schedule A increases the median survival time of responding patients but does not increase overall survival at 2 years.
2. The use of radiation first in a combined modality approach should be reconsidered.

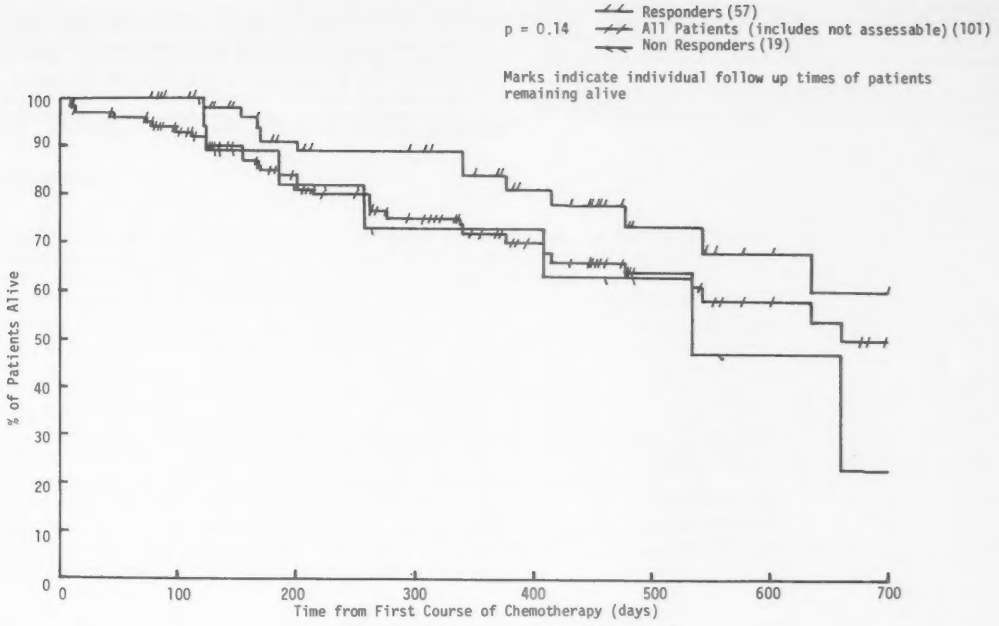
p = 0.14
Responders (57)
All Patients (includes not assessable) (101)
Non Responders (19)
Marks indicate individual follow up times of patients remaining alive
% of Patients Alive
Time from First Course of Chemotherapy (days)

FIGURE 4.—Actuarial survival of patients in Group I.

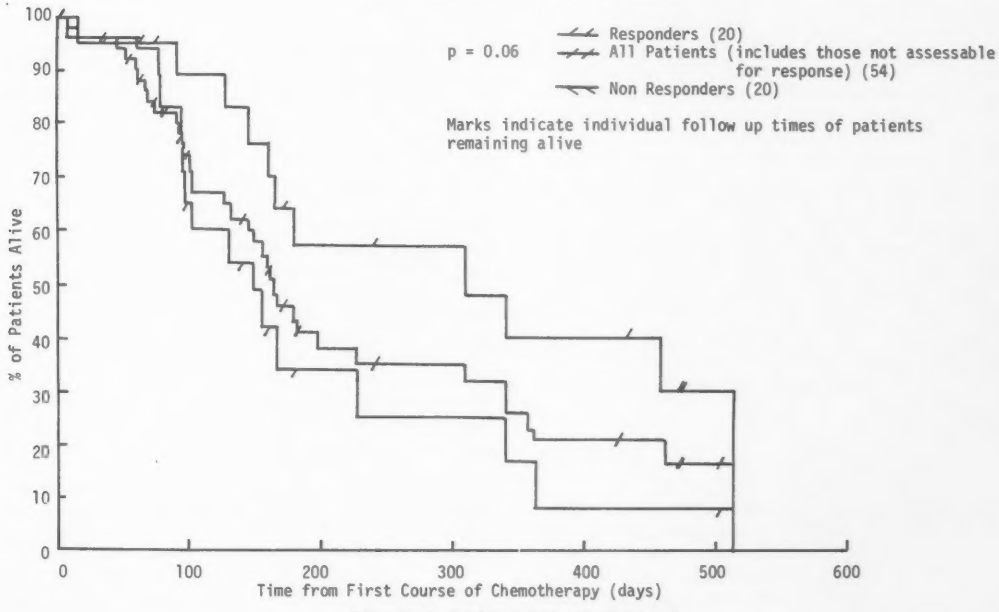
p = 0.06
Responders (20)
All Patients (includes those not assessable for response) (54)
Non Responders (20)
Marks indicate individual follow up times of patients remaining alive
% of Patients Alive
Time from First Course of Chemotherapy (days)

FIGURE 5.—Actuarial survival of patients in Group II.

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REFERENCES

1. Price LA. Liver damage and methotrexate. Br Med J 2:464, 1971.
2. Goldie JH, Price LA, and Harrap KR. Methotrexate toxicity: correlation with duration of administration, plasma levels, dose and excretion pattern. Eur J Cancer 8:409–414, 1972.
3. Price LA, Hill BT, Calvert AH, et al. Kinetically-based multiple drug treatment for advanced head and neck cancer. Br Med J 3:10, 1975.
4. Price LA, and Hill BT. A kinetically-based logical approach to the chemotherapy of head and neck cancr. Clin Otolaryngol 2:339–345, 1977.
5. Peto R, Pike MC, Armitgage, P, et al. Design and analysis of randomised clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J. Cancer 35:1, 1977.
6. Price LA, and Hill BT. Safe and effective combination chemotherapy for squamous cell carcinomas of the head and neck. J. Laryngol Otol 94:89, 1980.
7. O'Connor AD, Clifford P. Dalley VM, et al, Advanced head and neck cancer treated by combined radiotherapy and VTM cytotoxic regimen—four-year results. Clin Otolarngol 4:329–337, 1979.
8. Bezwoda WR, de Moor NG, and Derman DD. Treatment of advanced head and neck cancer by means of radiation therapy plus chemotherap[illegible]—a randomised trial. Med Paediatr Oncol 6:353, 1979.

Methotrexate Treatment of Advanced Head and Neck Cancers: A Dose Response Evaluation

R. L. Woods,[1]* R. M. Fox,[1] and M. H. N. Tattersall [1]

SUMMARY

Fifty-eight patients with advanced squamous cell cancer of the head and neck have been randomized on a study comparing three different weekly methotrexate (MTX) dose levels followed at 24 hours by standardized folinic acid rescue. Eighteen patients received MTX 5 g/m², 24 patients received 500 mg/m², and 16 patients received 50 mg/m². The initial response rates according to treatment were 50%, 21% and 31%, respectively. Six patients crossed from low- to medium-dose MTX after failing to respond (four) or relapsing, and one in each group had a tumor response. Eleven patients crossed over from initial medium-dose to high-dose MTX after failing to respond (ten) or relapsing, and one in each group had a tumor response. The median time to maximum response was 3.5 weeks. There were only two complete responders, and one is disease free more than 19 months after starting treatment and more than 15 months after stopping treatment.

The high-dose MTX treatment was significantly more toxic than lower doses, and there were four drug-related deaths (three in the high-dose group). The preliminary results of this study support the notion of a dose-response relationship to MTX in advanced squamous cell cancer of the head and neck.

[Cancer Treat Rep 65 (Suppl. 1):155–159, 1981]

Methotrexate (MTX) is the most widely used drug in the treatment of patients with advanced squamous cell cancers of the head and neck (1,2). Since the drug was first reported to be active in these tumors, a large number of different doses and schedules of treatment have been used, but there have only been two prospective clinical trials comparing two different MTX treatment schedules (3,4). In the past 5 years there have been several reports that massive-dose MTX treatment followed by folinic acid rescue (CF) 24 to 36 hours later has a better response rate than is usual with lower dose treatment (5–9). The advocates of this therapy acknowledge that this treatment program is potentially hazardous as well as expensive (8,10,11), but a number of surgical adjuvant studies have utilized this high-dose therapy and it is also being used in combination chemotherapy (1,12–15). At present no clear superiority has been demonstrated for massive-dose MTX compared to lower dose, cheaper, and less toxic treatment.

In February 1978, we initiated a prospective randomized controlled trial of weekly MTX using three different dose levels followed by standardized CF at 24 hours. The MTX doses were 50 mg/m², 500 mg/m², and 5 g/m². The protocol required that four weekly treatments be completed before evaluation of response. Responding patients continued the treatment for four treatments after achieving maximal response, or until progressive disease occurred, whichever came first. Patients failing to respond to the allocated treatment or relapsing on treatment crossed over to receive the next higher dose of MTX and continued treatment for a minimum of four courses before evaluation. Patients randomized initially to the higher MTX dose were removed from study if there was no response after four treatments. This paper reports a preliminary evaluation of the results of this study in the first 58 patients.

PATIENTS

All patients had histologically confirmed measurable advanced squamous cell cancer of the head and neck, and most had relapsed after initial surgery or radiotherapy. Some patients presented with locally advanced disease and received four courses of chemotherapy prior to receiving radiotherapy or surgery.

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There were 45 men and 13 women with a median age of 63 years (range, 15 to 83 years). Forty-one patients had had previous radiotherapy. All patients had a full clinical examination, including measurement of tumor and, in many cases, a photograph of the tumor, prior to starting chemotherapy. All patients had normal baseline serum creatinine, and, in addition, pretreatment cbc and biochemical screen, including liver function tests, were performed. Only patients with an ECOG performance status less than 3 were eligible to start chemotherapy.

There was no significant difference in the proportion of previously treated patients, nor in the performance status of patients in the three groups.

Table 1 shows the primary tumor sites of the 58 patients entered on trial, and also indicates which patients had had prior radiotherapy. Figure 1 shows the MTX-CF treatment regimen used in the study. It should be noted that all patients received the same pretreatment hydration, urinary alkalinization regimen, and post-MTX-CF program. This standardization reduces the possibility of any difference in the treatments being ascribed to differences in the method of drug administration or rescue.

Standard criteria for complete response (CR) and partial response (PR) were used to record results, and duration of response was measured from the start of treatment.

TABLE 1.—Initial disease sites in 58 patients

Site	No. patients	% patients	No. patients previously irradiated
Larynx	14	24	11
Floor of mouth	13	22	9
Tongue	8	14	6
Oropharynx	8	14	5
Other	6	10	3
Buccal mucosa	5	9	3
Nasopharynx	4	7	4
Total	58	100	41

RESULTS

Table 2 shows the initial randomization and indicates the number of patients responding to initial therapy. There is no significant difference in response rates between the regimens (chi squared test $P = <0.10$). Six patients crossed from the low- to medium-dose MTX treatment. Four of these were initial nonresponders, and one responded to the higher dose. Two patients who initially responded to low-dose MTX subsequently received medium-dose MTX after developing progressive disease, and one sustained a further response. Eleven patients crossed over from initial medium-dose MTX to high-dose

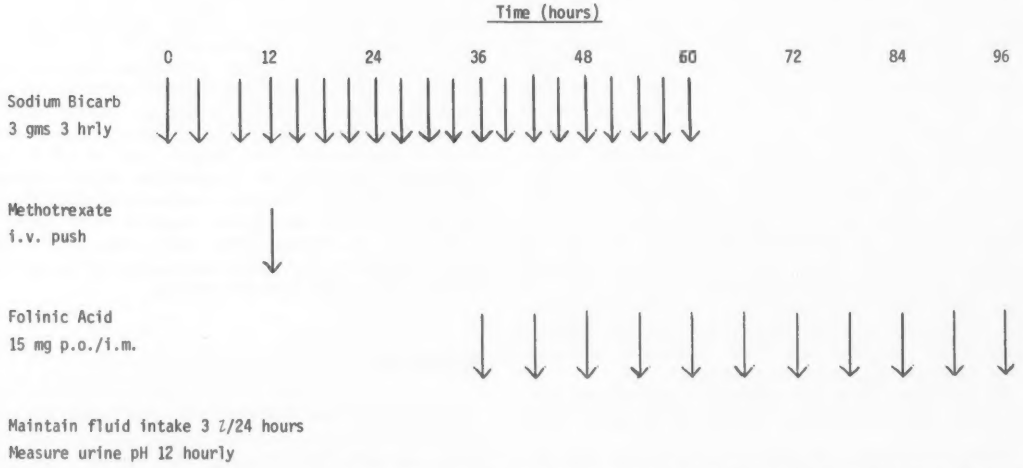

FIGURE 1.—MTX treatment program. Patients commenced urinary alkalinization (sodium bicarbonate 3 g orally at 3-hour intervals) and a high fluid intake (3 liters/24 hours) 12 hours before receiving the calculated dose of MTX as an iv injection. Urine pH was measured at 12-hour intervals and maintained >pH 7.0. CF (15 mg po/im/6 hours×12 doses) was started 24 hours after the MTX injection.

MTX. Ten patients were initially nonresponders to the medium-dose treatment, and two responded to the higher dose. One patient responded to medium-dose treatment and then relapsed, and one sustained a further response on the high-dose MTX. The cumulative response according to MTX dose is shown in table 3.

TABLE 2.—Initial randomization figures among the three MTX doses and number of responding patients

	MTX dose/m²/week		
	5 g	500 mg	50 mg
No. patients	18	24	16
No. responding	9	5	5
% response	50	21	31

TABLE 3.—Cumulative response to MTX dose (including dose escalation results)

	MTX dose/m²/week		
	5 g	500 mg	50 mg
No. of treatments	29	30	16
No. responding	12	7	5
% response	41	23	31

The mean time to maximum response was 3.5 weeks after start of chemotherapy (range, 1 to 8 weeks), and there was no obvious difference between regimens in the speed of response. Five of seventeen patients who had had no previous radiotherapy responded to MTX (29%), compared to 14 of 41 patients (34%) who had received previous radiotherapy.

The response to initial therapy by site is shown in table 4. The numbers in each category are too small at this stage to allow any conclusions to be drawn.

TABLE 4.—MTX responses according to site of primary tumor and initial dose

Site	MTX dose/m²/week			
	5 g	500 mg	50 mg	Total %
Tongue	3/3	1/3	0/2	50
Floor of mouth	2/5	1/3	2/5	38
Buccal mucosa	1/1	0/4	0/0	20
Oropharynx	0/1	1/4	1/3	25
Nasopharynx	0/1	0/2	1/1	25
Larynx	2/6	1/5	1/3	29
Other	1/1	1/3	0/2	33
Total	9/18	5/24	5/16	33

Figure 2 shows the survival according to initial therapy, and figure 3 shows the survival according to response to therapy. This includes responders following dose escalation, but does not include patients who received radiotherapy or surgery immediately after completing a trial of chemotherapy. It can be clearly seen that responders lived substantially longer than nonresponding patients. Of the two patients who achieved CR on initial therapy, one is alive and disease free more than 19 months after starting treatment, and more than 15 months after stopping treatment. At this stage, there is no significant difference in survival between the initial regimens.

The toxicity of MTX treatment according to dose is shown in table 5. The high-dose therapy is significantly more toxic than the medium or low doses (chi squared test $P=0.05$ for both). Three of the patients who developed renal toxicity did not become myelosuppressed. One of these patients was hemodialyzed when the creatinine and MTX levels were elevated 48 hours after MTX. The four fatal cases were myelosuppressed at the time of death.

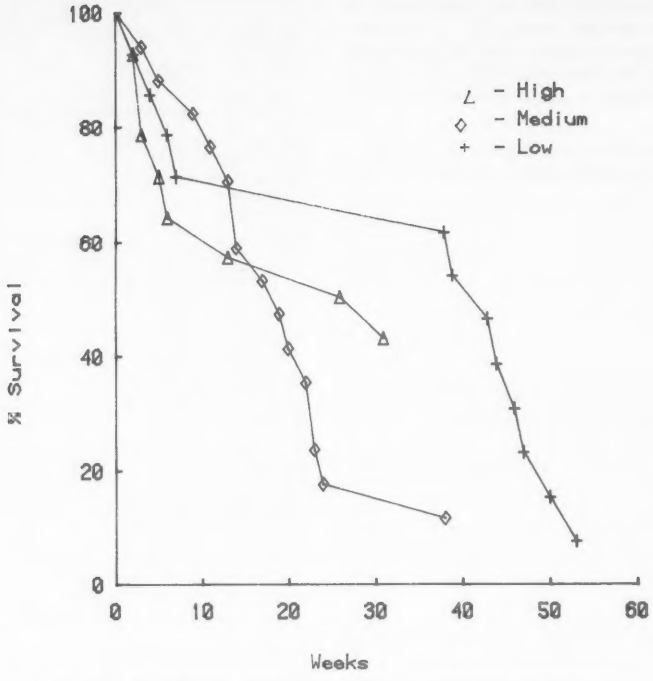

FIGURE 2.—Patient survival according to initial MTX treatment (dose/m²/week).

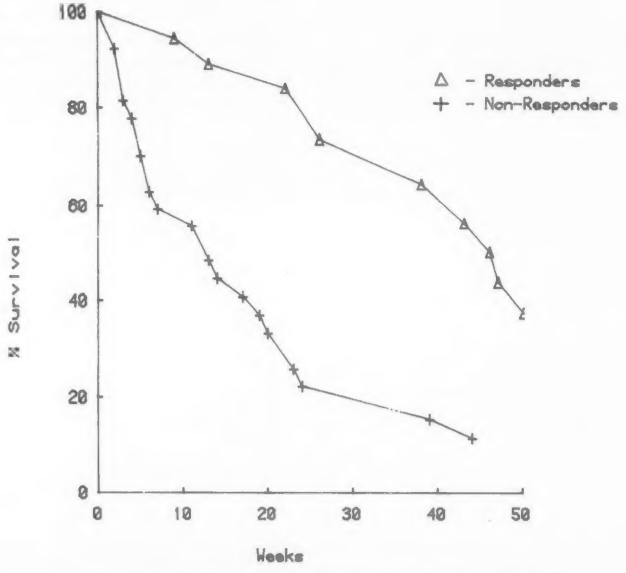

FIGURE 3.—Patient survival according to response to MTX treatment.

TABLE 5.—Toxicity of MTX treatment according to dose

Type of toxicity	MTX dose/m²/week		
	5 g	500 mg	50 mg
(ECOG grade 2 or worse)			
Myelotoxicity	8	3	1
Mucositis	4	3	1
Nausea and vomiting	6	1	1
Renal	6	0	1
Fatal	3	0	1
Chi squared: High dose versus Low dose or Medium dose	P = <0.05		

DISCUSSION

These preliminary data from a randomized controlled trial utilizing different initial doses of MTX suggest that high-dose MTX may have an improved response rate compared to treatment with lower doses. However, the results also indicate that patients who fail to respond to initial low-dose MTX may respond when the dose of MTX is increased, and that if they achieve a response to the higher dose treatment their overall survival may not be different from patients initially starting on the higher dose treatment. Thus, these results suggest that a standard approach for advanced squamous cell carcinoma of the head and neck using low-dose MTX as initial treatment may not be inferior to initial high-dose therapy provided that patients failing to respond to the low-dose treatment have the dose escalated after four courses. This strategy of management will reduce the risks of toxicity seen most frequently in patients receiving the high-dose treatment program, even though all patients receive iv fluids, urinary alkalinization, careful monitoring of renal function, and monitoring of MTX levels at 24 or 48 hours after treatment.

The low response rate (29%) in patients who have received prior radiotherapy is lower than that reported by others. In view of the small number of patients—17—in this group, however, little significance can be attributed to this.

Further analysis of the present trial is being undertaken, and it may be possible to identify those patients at greater risk of developing unacceptable toxicity associated with a high-dose MTX treatment program. Older patients may be particularly at risk.

REFERENCES

1. GOLDSMITH MA, and CARTER SK. The integration of chemotherapy into a combined modality approach to cancer therapy. V. Squamous cell cancer of the head and neck. Cancer Treat Rev 2:137, 1975.
2. BERTINO JR, MOSHER MB, and DECONTI RC. Chemotherapy of cancer of the head and neck. Cancer 31:1141, 1973.
3. LEVITT M, MOSHER MB, DECONTI RC, ET AL. Improved therapeutic index of methotrexate with "leucovorin rescue." Cancer Res 33:1729, 1973.
4. RICHARD JM, SANCHO H, BRUGERE J, ET AL. Intra-arterial methotrexate in head and neck tumours. Eur J Cancer 9:847, 1973.
5. LAHIRI SR, and PRASAD SC. High dose methotrexate and citrovorum factor rescue in lung and head and neck cancer. Proc Am Assoc Cancer Res and ASCO 14:101, 1974.
6. PITMAN SW, and FREI E (III). Weekly methotrexate citrovorum factor with alkalinization: tumour response in a phase II study. Proc Am Assoc Cancer Res and ASCO 18:124, 1977.
7. KIRKWOOD JM, MILLER D, PITMAN S, ET AL. Initial high dose methotrexate-leucovorin in advanced squamous carcinoma of the head and neck. Proc Am Assoc Cancer Res and ASCO 19:398, 1978.
8. BELL R, SULLIVAN JR, MOON WJ, ET AL. High-dose methotrexate therapy. Med J Aust 1:247, 1977.
9. KHANDEKAV JD, and WOLFF A. A clinical trial of high dose methotrexate with leucovorin rescue in advanced epidermal carcinoma of the head and neck. Proc Am Assoc Cancer Res and ASCO 18:281, 1977.
10. VON HOFF DD, PENTA JS, HELMAN LJ, ET AL. Incidence of drug-related deaths secondary to high-dose methotrexate and citrovorum factor administration Cancer Treat Rep 61:745, 1977.
11. DJERASSI I, KIM JS, NAYAK NP, ET AL. High-dose methotrexate with citrovorum factor rescue: a new approach to cancer chemotherapy. *In* Recent Advances in Cancer Treatment. (Tagnon HJ and Staquet MJ, eds). New York, NY, Raven Press, 1977, p 201.
12. PITMAN SW, MILLER D, WEICHSELBAUM R, ET AL. Weekly high dose methotrexate with leucovorin rescue as initial adjuvant therapy in advanced squamous cell carcinoma of the head and neck. *In* Adjuvant Therapy of Cancer. (Salmon SE and Jones SE, eds). Elsevier/North Holland Biomedical Press, 1977, p 467.
13. TARPLEY L, CHIETIEN PB, ALEXANDER C, ET AL. High dose methotrexate as a pre-operative adjuvant in the treatment of epidermal carcinoma of the head and neck. Am J Surg 130:481, 1975.
14. WEICHSELBAUM RR, MILLER D, PITMAN SW, ET AL. Initial adjuvant weekly high dose methotrexate with leucovorin rescue in advanced squamous carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 4:671, 1978.
15. BIANCA A, TAYLOR SG (IV), REICH S, ET AL. Combination chemotherapy pilot studies in head and neck squamous cell cancer. Cancer Treat Rep 63:158, 1979.

Methotrexate in the Chemotherapy of Lung Cancer

J. F. Smyth[1]* and H. T. Ford [1]

Summary

Methotrexate (MTX) is an active agent in both non-small cell and small cell anaplastic lung cancer. A 26% partial response rate was obtained in 27 previously untreated patients with squamous cell bronchogenic tumors treated with 24-hour infusions of MTX at 400 mg/m². Responses were seen predominantly in extrathoracic sites. In 38 evaluable patients with small cell carcinomas, 24-hour infusions of MTX at 200 mg/m² in combination with cyclophosphamide and CCNU produced a 43% complete remission (CR) rate for a median survival of 62 weeks. Fifty-six percent of the CR's were seen in patients with extensive disease.

[Cancer Treat Rep 65 (Suppl. 1):161–163, 1981]

It is now well recognized that the term "carcinoma of the bronchus" refers to a number of distinct disease entities classified on the basis of their histological appearance, and characterized by marked differences in biological behavior and response to treatment. The response to chemotherapy covers a spectrum ranging from the highly refractory adenocarcinoma of the bronchus to the very sensitive small cell anaplastic (oat cell) carcinoma. The use of combination chemotherapy has proved relatively ineffectual for non-oat cell bronchogenic carcinoma, and current research is directed at a reappraisal of single agent data for these diseases. Conversely the proven sensitivity of oat cell lung cancer to many different single agents has resulted in the use of combinations that produce complete remissions (CR) resulting in significant prolongation of survival.

NON-OAT CELL BRONCHOGENIC CARCINOMA

The response of non-oat cell bronchogenic carcinoma to methotrexate (MTX) has been evaluated in a number of studies using different doses and methods of administration (1–4) as shown in table 1. Response rates vary from 0% to 100%, although the study by Djerassi (2) was performed on only six patients and clearly has not been substantiated by later, more extensive evaluations of high-dose regimens.

TABLE 1.—Objective response of non-oat cell bronchogenic carcinoma to MTX

Regimen	No. patients	% response	Ref.
0.6 mg/kg im 2 × week	23	35	1
0.2 mg/kg im 2 × week	11	9	1
1 to 18 g iv 6 hours with folinic acid	6	100	2
1.7 to 7.5 g/m² iv 6 hours with folinic acid	28	20	3
200 mg po 6 hours × 4 with folinic acid	13	0	4
400 mg/m² iv 24 hours with folinic acid	27	26	-

Included in table 1 are data from an investigation performed at the Royal Marsden Hospital, Sutton, United Kingdom, investigating the effects of 24-hour infusions of MTX at 400 mg/m², followed by folinic acid at 12 mg/m² q 6 hours × 8. The study was designed to evaluate the response to micromolar plasma concentrations of MTX. Twenty-seven untreated patients with unresectable squamous cell carcinoma of the bronchus, none of whom had received prior radiotherapy or chemotherapy, received a minimum of three MTX infusions at 3-week intervals. Measurement of MTX concentration in 19 of these patients confirmed mean plasma levels of 3×10^{-6} M at 2 hours, 8×10^{-6} M at 18 hours, and 1×10^{-5} M at 24 hours, from the start of MTX infusion. A partial response (PR) was documented in 7 of 27 patients (26%) where all but one of these responses were seen in extrathoracic disease. Sites of response were as follows: a response of the primary lesion in the chest was seen in one patient, there were three PR's involving lymph nodes, and four in the liver—as measured clinically, by biochemical assessment and iso-

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tope liver scan. The mean duration of these responses was 3.6 months (±2.9).

OAT CELL BRONCHOGENIC CARCINOMA

MTX has been used extensively in the chemotherapy of oat cell carcinoma of the bronchus, most frequently in combination with cyclophosphamide and CCNU. In a series of consecutive studies performed at the NCI-VA Medical Oncology Branch, MTX was evaluated at a dose of 10 mg/m² po biweekly together with cyclophosphamide (500 mg/m² iv Days 1 and 22) and CCNU (50 mg po Day 1) against a more intensive regimen of 15 mg/m² MTX with double the amounts of cyclophosphamide and CCNU (5). None of the 9 patients treated with the less intensive regimen achieved CR, whereas 7 of 23 patients (30%) treated with the higher doses achieved CR for a median survival of 10.5 months. Subsequently 50 patients (6) were treated with the same doses of MTX and CCNU, but with the cyclophosphamide increased to 1,500 mg/m² iv, in a study where these three drugs were alternated with vincristine, Adriamycin, and procarbazine, a non-cross-resistant combination (VAP) (7). The CR rate was increased to 56% for a median overall survival of 61 weeks. Thirty-four percent of these 50 patients had limited disease.

At the Royal Marsden Hospital, Sutton, United Kingdom, we performed a trial based on the last-mentioned NCI-VA trial, where the major difference in protocol design related to the use of MTX. Table 2 shows the MTX, cyclophosphamide, and CCNU regimen (MCC): cyclophosphamide and CCNU were combined with a 24-hour infusion of MTX at 200 mg/m², followed by folinic acid rescue. Two 6-week cycles of MCC were administered in the induction phase, followed by monthly courses of VAP, for a total treatment period of approximately 9 months. Response was assessed at 12 weeks. The study was designed to randomize responding patients either to receive maintenance chemotherapy alone, or to receive chemotherapy following radiotherapy to the original primary area in the chest (4,000 rad in 4 weeks). Of 42 patients entered into this trial, 37 are assessable, 27 of whom (73%) had extensive disease as determined by clinical examination, routine biochemistry, bone marrow aspiration, and isotope scans of brain, liver, and bone. An objective response was seen in 29 of the 37 assessable patients (78%); 16 of the 37 (43%) had CR. Of the 16 patients with CR, 9 had extensive and 7 had limited disease (table 3).

The median survival for all 37 assessable patients was 39 weeks, with the 16 CR's surviving to 62 weeks, PR's surviving to 38.5 weeks, and the patients who failed to respond surviving to a median of 17.5 weeks.

TABLE 2.—MCC oat cell regimen

MCC	MTX	200 g/m² iv over 24 hours + rescue q 3/52 × 4	
	CTX	1 g/m² iv stat q 3/52 × 2, then 750 mg/m² q 3/52 × 2	
	CCNU	100 mg/m² po stat, then 50 mg/m² po at 6/52 9.04	
VAP	VCR	1.4 mg/m² iv Days 1 and 8	q 28 days
	ADR	40 mg/m² iv Day 1	
	PCZ	100 mg/m² po Days 1-14	

A 12-week course of MCC is followed by 6 monthly cycles of VAP.

TABLE 3.—MCC oat cell trial: response by extent of disease

	Limited	Extensive
CR	7/16 (44%)	9/16 (56%)
PR	2/13 (15%)	11/13 (85%)
No Response	1/8 (12%)	7/8 (88%)

The survival of complete and partial responders versus those who failed to respond is significant at P <0.03 (Gehan).

The addition of radiotherapy had no influence on survival, but there were more relapses at the primary site in patients receiving chemotherapy alone. The brain was the site of recurrent disease in 21% of the responding patients.

Response to induction therapy was not influenced by age, sex, performance status, or extent of disease, but good initial performance status correlated positively with duration of response (P <.003) and survival (P <.005).

DISCUSSION

MTX is an active agent in squamous and oat cell lung cancer. For squamous cell carcinomas, 24-hour infusions achieving micromolar plasma concentrations of MTX did not result in a higher response rate than that obtained by the previously described low-dose biweekly M administration of 0.6 mg/kg (1). However, variations in the site of response have not been described previously. The predominance of extrathoracic responses reported in the present study—in patients who had not received radiotherapy to the chest—is of relevance to the design of future treatment policies that may combine chemotherapy with radiotherapy to the primary lesion.

For oat cell bronchogenic carcinoma, MTX is one of many active single agents that, in combination, can result in CR's that prolong survival. In the present trial, the use of 24-hour infusions of MTX at 200 mg/m² in combination with cyclophosphamide and CCNU

resulted in a 43% CR rate for a median survival of 62 weeks. In comparing the present study with the previously reported series of studies from the NCI-VA group, in which induction treatment utilized MTX at 15 mg/m² orally biweekly, there is little difference in the overall response rate, and particularly in the proportion of patients attaining CR. The fact that 56% of the CR's in the present study were seen in patients with extensive disease is encouraging but set against this is the observation that the more intensive use of MTX for induction therapy had little, if any, effect on the incidence of CNS metastases, where the brain was the site of recurrent disease in 21% of the responding patients. There remains no statistical evidence that the addition of radiotherapy to chemotherapy influences overall survival in small cell bronchogenic carcinoma, but it is likely that MTX, which is well suited to combination with other drugs and with radiotherapy, will continue to be evaluated in future trials of therapy for this disease.

REFERENCES

1. Selawry O, Krant M, Scotto J, et al. Methotrexate compared with placebo in lung cancer. Cancer 40:4–8, 1977.
2. Djerassi I, Rominger CJ, and Sun Kim J. Phase I study of high doses of methotrexate with citrovorum factor in patients with lung cancer. Cancer 30:22–30, 1972.
3. Ettinger DS, Stanley KE, and Nystrom JS. High dose methotrexate with citrovorum factor rescue in inoperable non-oat cell bronchogenic carcinoma. Proc Am Soc Clin Oncol 15:C–299, 1979.
4. Tornyos K, and Faust H. Oral high dose methotrexate with citrovorum rescue in metastatic squamous cell carcinoma of the lung. Cancer 41:400–404, 1978.
5. Cohen MH, Creaven PJ, Fossieck BE, et al. Intensive chemotherapy of small cell bronchogenic carcinoma. Cancer Treat Rep 61:349–354, 1977.
6. Bunn PA, Cohen MH, Ihde DC, et al. Advances in small cell bronchogenic carcinoma. Cancer Treat Rep 61:333–342, 1977.
7. Cohen MH, Broder LE, Fossieck BE, et al. Combination chemotherapy with vincristine, Adriamycin and procarbazine in previously treated patients with small cell carcinoma. Cancer Treat Rep 61:485–487, 1977.

Chemotherapy of Bladder Cancer: A Review

Philip H. Smith [1, 2*]

SUMMARY

Cytotoxic chemotherapy is used increasingly in the management of bladder cancer. The agents used, intravesically and systemically, are reviewed, together with preliminary reports of certain trials of adjuvant chemotherapy that have now been activated.

[Cancer Treat Rep 65 (Suppl. 1):165–173, 1981]

Bladder cancer, the second most common of the urological tumors, is of increasing interest to medical oncologists, in part because of the demonstration of the potential value of cytotoxic chemotherapy in this condition, and in part because of the increasing dissatisfaction of surgeons and radiotherapists with the results of conventional therapy. Although chemotherapy may be of benefit to all patients, its effects are easiest to assess in those with noninvasive (category T1) and deeply invading (category T3) lesions (1). It is in the latter group that the major interest of this review lies, but each group deserves separate consideration.

CHEMOTHERAPY OF NONINVASIVE BLADDER CANCER

For the patient with noninvasive bladder cancer, the standard treatments of transurethral resection (TUR) or cystodiathermy usually control the presenting lesion(s), but recurrences are seen in 60% of patients (2). All patients require life-long followup by cystoscopy and repeated iv pyelography.

In a minority of patients, frequent and extensive recurrences may occur, rendering control by TUR very difficult. Several additional treatments have been suggested as adjuvants to TUR in the hope of avoiding cystectomy, with all its disadvantages. These include bladder distention therapy (3), mucosal denudation (4), and treatment with vitamins A, B_6, and C (5–7). However, most reports have concerned the use of either radioactive isotopes or cytotoxic agents by intravesical instillation. Some of the agents used are listed in table 1. Initial interest in silver nitrate or podophyllin was superseded by radioactive isotopes, but the isotopes were not generally effective and were commonly followed by bladder fibrosis and contracture. Of the cytotoxic agents, extensive experience is available from only three: Adriamycin (ADM), Epodyl®, and thiotepa. Combined results of various studies using these agents are summarized in table 2.

TABLE 1.—Agents used intravesically for the control of category T1 bladder cancer (22–24)

Silver nitrate	CTX
Podophyllin	Daunomycin
Isotopes:	
Yttrium 90	Epodyl®
Cobalt 60 beads	
Gold 198	5FU
Arsenic 76	Mannitol myleran
Altinomycin D	Mechlorethamine
ADM	Peptichemio
BLM	Thiotepa
Chlorpactin	Trenimon
Cis-platinum	VM-26

TABLE 2.—Combined statistics of the use of ADM, Epodyl®, and thiotepa in the prophylaxis and treatment of category T1 bladder cancer (6,8,9,22–47)

	Prophylaxis		Therapy	
	Patients	Recurrence free	Patients	Remissions (CR + PR)
ADM	62	40 (65%)	411	239 (58%)
Epodyl®	–	–	167	136 (81%)
Thiotepa	167	98 (59%)	266	166 (62%)

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[2] I should like to acknowledge my thanks to the members of the EORTC Urological Group and the YUCRG who have entered patients into the studies quoted, to the Medical Illustrations Department of St. James's University Hospital, Leeds, for the preparation of figure 1, and to Miss S. A. Barrowby for her help in the preparation of this paper and for typing the manuscript.

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Although beneficial effects are similar, their toxicities differ, at least in our own experience, which is limited to Epodyl® and thiotepa. Despite Riddle's favorable comments (8), the Yorkshire Urological Cancer Research Group (YUCRG) found a high incidence of toxicity with Epodyl® in terms of chemical cystitis and bladder contracture (9). In the Urological Group of the European Organization for Research on the Treatment of Cancer (EORTC), we have compared intravesical thiotepa 30 mg, VM-26 50 mg, and control (Protocol 30751). The toxicity of thiotepa (table 3) has so far been minimal. It should be noted that it differs little from that seen in the control group.

TABLE 3.—Toxicity of intravesical thiotepa 30 mg and control in EORTC protocol 30751

	Thiotepa (89 patients)	Control (82 patients)
Wbc < 4,500	2	2
Platelets <150,000	14	10
Hb < 11G	1	2
Wbc < 4,500 *and* Platelets < 150,000	4	-
Hb < 11G *and* Wbc < 4,500	1	-
Hb < 11G *and* Platelets <150,000	-	2
Treatment stopped because of toxicity	3	-

The EORTC Urological Group has a continuing interest in the use of intravesical chemotherapy in patients with category T1 bladder cancer, and implemented its first study (Protocol 30751) in 1975. Recruitment to this study is now complete, and the preliminary evaluation has demonstrated the superiority of thiotepa in lowering the recurrence rate per 100 patient months—thiotepa 6.93, VM-26 10.12, control 9.97 (10). Table 4 shows the outlines of this protocol and of the studies that have replaced it.

Of the current EORTC studies, two are intravesical and one oral. Protocol 30782 is of particular interest to members in Belgium, France, Italy, and Spain, while Protocol 30790 will probably be restricted to participants in Germany, Holland, and the United Kingdom because of the limited general availability of Epodyl®. The oral study follows the paper by Byar et al (6), and is designed to investigate the value of oral pyridoxine. Patients have tryptophan load tests before treatment and after 6 months to evaluate the incidence and significance of abnormalities of tryptophan metabolism. The laboratory tests are being carried out in one laboratory in Pontefract, England, and because of this, entry into the study is restricted to surgeons in the United Kingdom. Treatment is administered on a double blind basis. It is hoped that preliminary evaluations of these three studies will be available within 2 years.

Our experience in the last 3 years in the EORTC has convinced us that intravesical chemotherapy is time consuming for both patient and doctor, and is probably best reserved for those with recurrent disease, preferably for those with multiple lesions. We have also become increasingly aware of the great amount of work still to be done not only in terms of choice of drug and dose but also in determining the correct concentration, volume of administration, patient position, time of commencement of treatment, and frequency and duration of treatment.

Following our initial study, we regard thiotepa as the standard drug against which other intravesical agents must be judged because of its known effectiveness with minimal toxicity. Obviously, oral therapy would be preferable, whether with pyridoxine or with methotrexate (MTX), a regimen which is to be discussed in these proceedings.[3]

CHEMOTHERAPY OF INVASIVE BLADDER CANCER

The choice of treatment for the patient with invasive bladder cancer is not easy, and the alternatives have recently been well reviewed by Grossman (11). The use of preoperative radiotherapy and cystectomy seems to offer the best chance of survival for those fit for cystectomy, since 5-year survival after this combination is 40% to 50%, while after radical radiotherapy it is only 20% to 30%. This approach has been considered in detail by Wallace and Bloom (12), Prout (13), Miller (14), and Whitmore and Batata (15).

Patients selected for these studies were fit for cystectomy, but despite this, for category T3 patients: (a) 40% were dead within 1 year; (b) 5-year survival rarely exceeded 49%; and (c) metastatic disease was the first sign of failure in 36% of patients after cystectomy, the lesions declaring themselves within the first year in 85% of these patients.

The improvement of 10% to 20% in 5-year survival is obtained at the cost of cystectomy in 100% of the patients. Of these, it is unnecessary in the 30% who would survive following radiotherapy alone, and useless in the 40% who still die within 1 year. These statistics offer little comfort to surgeon or radiotherapist, and emphasize the importance of developing some effective additional or alternative therapy.

The high early death rate and the known high incidence of metastases as a cause of failure of treatment suggest that cytotoxic chemotherapy may have an important role to play. Its effect may be limited by the kinetic factors so well described by Anderson (16), but this should not deter active investigation of the possibilities that undoubtedly exist.

Considerable progress has been made, almost all within this decade, and this report will summarize the information available on the use of systemic cytotoxic chemotherapy by single agents, with combination regimens, in the adjuvant situation, and finally mention some of the phase III trials now in progress.

[3] R. R. Hall et al. Oral methotrexate therapy for multiple superficial bladder carcinomata. Cancer Treat Rep 65 (Suppl 1): 175–178, 1981.

SINGLE AGENT CHEMOTHERAPY

Information is now available from phase II studies on the use of 12 agents (table 4). Most produce remission rates of 30% to 40%. Many of the agents, including ADM, bleomycin (BLM), and cis-diamminedichloroplatinum (DDP), have major toxic side effects that render them unsuitable for some patients and limit their duration of use in all. Others, including cyclophosphamide (CTX), 5-fluorouracil (5FU), and MTX, are relatively nontoxic and can, if desired, be administered orally over prolonged periods. Even in the absence of objective remission, symptomatic relief is commonly found.

TABLE 4.—Remission rate in phase II studies of single agents in patients with invasive bladder cancer (36, 48–53)

Drug	Remission rate (CR + PR/ evaluable patients)
ADM	45/182 (25%)
BLM	13/56 (23%)
Cis-platinum	30/74 (41%)
CTX	14/40 (35%)
5FU	25/72 (35%)
MTX	46/157 (29%)
MITO-C	25/75 (33%)
NCS	15/36 (42%)
Pala	0/14 (0%)
Vinblastine	1/10 (10%)
Vincristine	3/15 (20%)
VM-26	11/64 (17%)

COMBINATION THERAPY

Many of the effective agents have been combined in the hope of producing an additive or synergistic effect, but table 5 shows that such combinations have so far produced only an extra 10% incidence of remissions. With such regimens toxicity is likely to limit the duration of use after four or more cycles have been given (17), a problem that still remains to be solved, perhaps by a combination of induction and maintenance regimens.

ADJUVANT CHEMOTHERAPY

At least six regimens of adjuvant chemotherapy have now been reported, and these are summarized in table 6. Although Martinez-Pineiro (18) found that the morbidity and mortality were unacceptably high in his regimen, which included radical cystectomy, Merrin (19) and Merrin and Beckley (20) found no such problems. In the YUCRG study (17), all patients accepted four cycles of chemotherapy, but thereafter patients increasingly declined further treatment. Socquet (21), however, using MTX, has experienced no such problems, and has also found a very high survival rate (fig 2).

TABLE 5.—Remission rate in phase II studies of combination regimens in patients with invasive bladder cancer (49,52,54–64)

Drug	Remission rate (CR + PR/ evaluable patients)
ADM + CTX	12/36 (33%)
ADM + DDP	14/26 (54%)
ADM + 5FU	33/83 (40%)
ADM + VM-26	5/19 (26%)
CTX + DDP	17/35 (49%)
ADM + CTX + DDP	34/73 (47%)
ADM + DDP + 5FU	18/44 (41%)
CTX + OHU + VBL + VCR	4/18 (22%)

PHASE III TRIAL OF ADJUVANT CHEMOTHERAPY

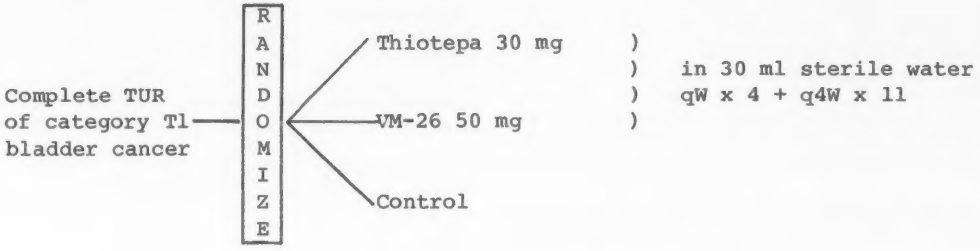
Figure 3 shows the YUCRG and EORTC protocols for adjuvant chemotherapy for patients with category T3M0 bladder cancer.

In the YUCRG study, patients with T3NXM0 bladder cancer receive radical radiotherapy as the primary treatment. At diagnosis they are randomized to receive either no additional treatment or ADM and 5FU following radiotherapy. Although 93 patients have now been entered, recruitment is likely to take a further 12 to 18 months, after which a preliminary evaluation can be undertaken.

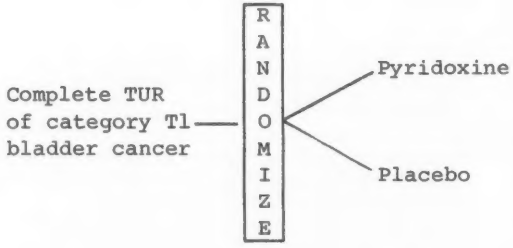
The EORTC Urological Group has recently implemented a study for patients with category T3N0–2M0 lesions to compare no additional treatment versus 5FU and ADM following primary treatment by flash radiotherapy and radical cystectomy, largely in France and Spain, and following radical cystectomy alone in Italy. Since June 1979, four patients have been entered in this study.

These two trials are among the first to incorporate adjuvant chemotherapy with agents known to be effective at the doses used, and the results are urgently required to allow a preliminary evaluation of the potential of adjuvant chemotherapy.

Figure 1.--EORTC Urological Group protocols for the treatment of category T1 bladder cancer

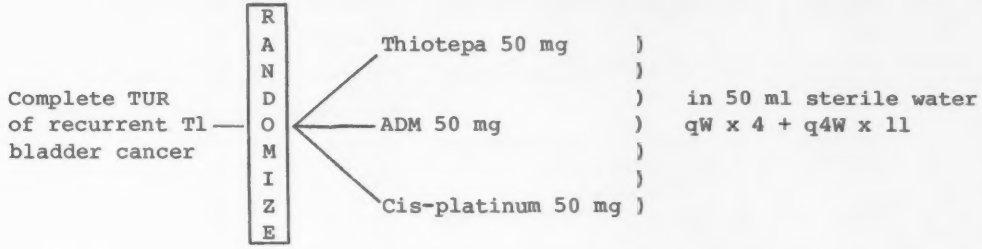


Protocol 30751. A comparison of intravesical thiotepa, VM-26, and control. Study Coordinator: Dr. C. C. Schulman, Brussels, Belgium. Activated November 1975. Recruitment completed November 1978.

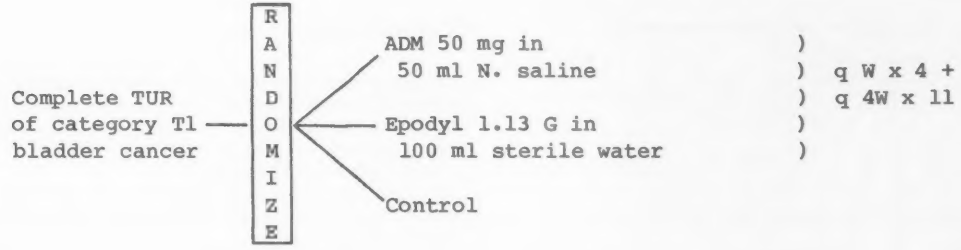


Protocol 30781. A comparison of oral pyridoxine (20 mg/day) and placebo. Study Coordinator: Mr. M. R. G. Robinson, Pontefract, England. Activated January 1979; 71 patients entered by October 1979. A double blind study with tryptophan load tests before and after 6 months of treatment.

Figure 1. (continued)



Protocol 30782. A comparison of intravesical thiotepa, ADM, and cis-platinum. Study Coordinators: Dr. C. C. Schulman, Brussels, Belgium; Professor M. Pavone-Macaluso, Palermo, Sicily. Activated March 1979; 46 patients entered by October 1979.



Protocol 30790. A comparison of intravesical ADM, Epodyl®, and control. Study Coordinator: Dr. K. Kurth, Rotterdam, Holland. To be activated November 1979.

TABLE 6.—Regimens used in adjuvant chemotherapy for patients with invasive bladder cancer (17–21)

Author	Patients	Radiotherapy	Surgery	Chemotherapy	Immunotherapy
Martinez-Pineiro	10	4,000 to 6,000 rad.	NIL/TUR/diversion	ADM 50 mg/m² 5FU 500 mg/m²	q 21 BCG 150 mg q W × 4 + q 4W
Martinez-Pineiro	10	4,000 rad	Radical cystectomy	ADM 50 mg/m² 5FU 500 mg/m²	q 21 BCG 150 mg q W × 4 + q 4W
Merrin and Beckley	25	NIL	Radical cystectomy	ADM 40 mg/m² CTX 200 mg/m² q D × 4	q 21 NIL
Merrin	21	NIL	Radical cystectomy	DDP 50 mg/m² q W × 6 then q 3W	NIL
Socquet	33	NIL	Partial (25) or total (8) cystectomy.	MTX 1,000 mg + CF then MTX 2,000 mg q 3W for 6 months at least	NIL
YUCRG	18	4,500 to 6,000 rad.	NIL/TUR	ADM 50 mg/m² 5FU 500 mg/m² minimum 4 cycles	q 3W NIL

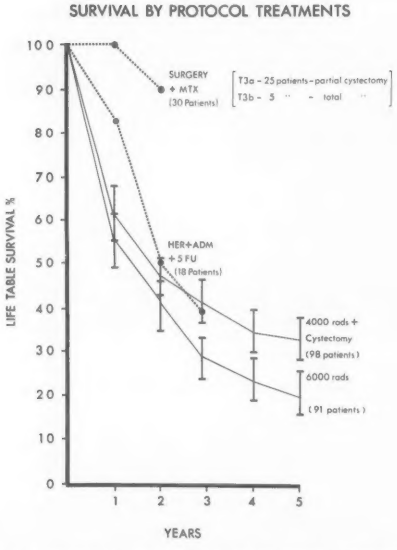

SURVIVAL BY PROTOCOL TREATMENTS
LIFE TABLE SURVIVAL %
YEARS
SURGERY + MTX (30 Patients)
T3a - 25 patients - partial cystectomy
T3b - 5 " - total "
HER+ADM + 5 FU (18 Patients)
4000 rads + Cystectomy (98 patients)
6000 rads (91 patients)

FIGURE 2.—Survival following radical radiotherapy, preoperative radiotherapy, and cystectomy, radical radiotherapy with adjuvant 5-FU and ADM, and partial or total cystectomy with adjuvant MTX (modified from Wallace and Bloom 1976 by kind permission of the British Journal of Urology) (12). The survival of the 5-FU/ADM patients is taken from Smith et al (53), and that of the adjuvant MTX patients from Socquet (21).

CONCLUSIONS

There has been an increasing interest in the role of cytotoxic chemotherapy in the management of bladder cancer within the last 10 years. From the studies so far reported it is possible to conclude that:

A. For intravesical therapy

(i) Thiotepa, ADM, and Epodyl®, are similarly effective when instilled intravesically in patients with category T1 disease.

(ii) Of these agents, thiotepa is almost certainly the least toxic.

B. For systemic therapy

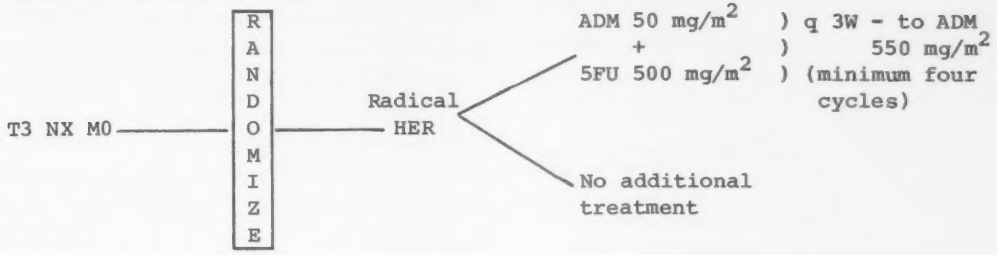
(i) In phase II studies of 12 agents, 30% to 40% remission rates have been obtained with 6 agents—DDP, CTX, 5FU, MTX, mitomycin C (MITO-C), and neocarzinostatin (NCS). Of these, MTX is one of the least toxic and may be administered for prolonged periods of time (65).

(ii) Of the combination regimens tested, five produce remissions of 40% to 50%.

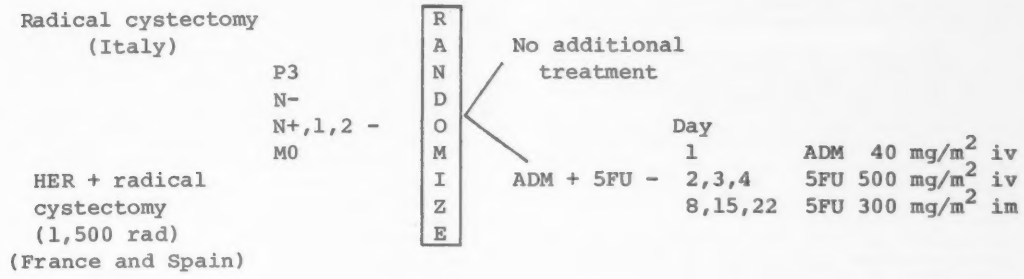
(iii) Adjuvant chemotherapy has been safely given in five separate regimens. Of these, two, involving ADM and 5FU in one and MTX in the second, offer hope of prolonged survival.

(iv) At least three randomized trials of adjuvant chemotherapy are now in progress using ADM + 5FU in two and MTX in one.

Figure 3.--Schema of YUCRG and EORTC protocols for adjuvant chemotherapy in patients with category T3 M0 bladder cancer



YUCRG Protocol 771. Study Coordinator: Mr. B. Richards, York, England. Activated April 1977; 96 patients entered to October 1979.



EORTC Protocol 30784. Study Coordinator: Professor J. A. Martinez-Pineiro, Madrid, Spain. Activated June 1979.

REFERENCES

1. UICC TNM Classification of Malignant Tumors, 2d ed, Geneva, Imprimerie G. de Buren S.A., 1974.
2. Pavone-Macaluso M. Chemotherapy of vesical and prostatic tumors. Br J Urol 43:701–708, 1971.
3. Helmstein K. Treatment of bladder carcinoma by hydrostatic pressure technique: report of 43 cases. Br J Urol 44:434–450, 1972.
4. Lund F. Mucosal denudation (stripping) for bladder papillomatosis. Scand J Urol Nephrol 3:204–207, 1969.
5. Bishop MC. The treatment of bladder cancer with vitamin A. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
6. Byar D, Blackard C, and Vacurg. Comparisons of placebo, pyridoxine and topical thiotepa in preventing recurrence of stage I bladder cancer. Urology 10:556–561, 1977.
7. Schlegel JU, Pipkin GE, Nishimura R, et al. The role of ascorbic acid in the prevention of bladder tumor formation. J Urol 103:155–159, 1970.
8. Riddle PR. The management of superficial bladder tumors with intravesical Epody®. Br J Urol 45:84–87, 1973.
9. Robinson MRG, Shetty MB, Richards B, et al. Intravesical Epodyl® in the management of bladder tumors: combined experience of the Yorkshire Urological Cancer Research Group. J Urol 118:972–973, 1977.
10. Schulman CC, and EORTC Urological Group. Adjuvant therapy of T1 bladder carcinoma: preliminary results of an EORTC randomized study. *In* Bladder Tumors and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
11. Grossman HB. Current therapy of bladder carcinoma. J Urol 121:1–7, 1979.
12. Wallace DM, and Bloom HJG. The management of deeply infiltrating (T3) bladder carcinoma: controlled trial of radical radiotherapy versus pre-operative radiotherapy and radical cystectomy (first report). Br J Urol 48:587–594, 1976.
13. Prout GR (jr). The role of surgery in the potentially curative treatment of bladder carcinoma. Cancer Res 37:2764–2770, 1977.
14. Miller LS. Preoperative irradiation for bladder cancer: the 2000 versus 5000 rad controversy. *In* Cancer of the Genitourinary Tract (Johnson DE, and Samuels ML, eds). New York, NY, Raven Press, 1979, pp 81–88.
15. Whitmore WF, and Batata, MA. Preoperative irradiation with cystectomy for bladder cancer. *In* Cancer of the Genitourinary Tract (Johnson DE, and Samuels ML, eds). New York, NY, Raven Press, 1979, pp 89–100.
16. Anderson T. Developmental concepts: effective chemotherapy for bladder carcinoma. Semin Oncol 6:240–248, 1979.
17. Glashan RW, Houghton AL, Robinson MRG, et al. A toxicity study of the treatment of category T3 bladder tumours with a combination of radiotherapy and chemotherapy. Br J Urol 49:669–672, 1977.
18. Martinez-Pineiro JA. Integrated therapy for invasive bladder carcinoma. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
19. Merrin C. Adjuvant post surgical chemotherapy with cyclophosphamide doxorubicin hydrochloride and cis diammine-dichloroplatinum in patients with bladder cancer. *In* Bladder Cancer–Principles of Combination Therapy (Hendry WF, Oliver RTD, and Bloom HJG, eds). London, Butterworth & Co, 1979. In press.
20. Merrin C, and Beckley S. Adjuvant chemotherapy for bladder cancer with doxorubicin hydrochloride and cyclophosphamide: preliminary report. J Urol 119:62–63, 1978.
21. Socquet Y. Surgery and adjuvant chemotherapy with high-dose methotrexate and folinic acid rescue for infiltrating tumors of the bladder. Cancer Treat Rep 65 (Suppl 1): 187–189, 1981.
22. Pavone-Macaluso M. Intravesical treatment of superficial (T1) urinary bladder tumours–a review of a 15-year experience. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 21–36.
23. Jacobi GH, Kurth KH, Klippel KF, et al. On the biological behaviour of T1-transitional cell tumours of the urinary bladder and initial results of the prophylactic use of topical Adriamycin under controlled and randomised conditions. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 83–94.
24. Abbassian A, and Wallace DM. Intracavitary chemotherapy of diffuse non-infiltrating papillary carcinoma of the bladder. J Urol 96:461–465, 1966.
25. Izbicki RM, Pontes E, and Vaitkevicius VK. Adriamycin bladder instillation. *In* Proceedings of the 12th Annual Meeting of the American Society of Clinical Oncology. Toronto, May 4–8, 1976.
26. Csellar M, and Csontai A. Local application of thiotepa in the prevention of recurrent papillary carcinoma of the bladder. Int Urol Nephrol 11(1):39–44, 1979.
27. Burnand KG, Boyd PGR, Mayo ME, et al. Single-dose intravesical thiotepa as adjuvant to cystodiathermy in the treatment of transitional cell bladder carcinoma. Br J Urol 48:55–59, 1976.
28. Veenema RJ, Dean AL (jr), Uson AC, et al. Thiotepa bladder instillations: therapy and prophylaxis for superficial bladder tumours. J Urol 101:711–715, 1969.
29. Neih PT, Daly JJ, Heaney JA, et al. The effect of intravesical thiotepa on normal and tumor epithelium. J Urol 119:59–61, 1978.
30. Wescott JW. The prophylactic use of thiotepa in transitional cell carcinoma of the bladder. J Urol 96:913–918, 1966.
31. Nilsson S, Eriksson O, Johansson S, et al. Intravesical therapy with Adriamycin in carcinoma in situ. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 79–81.
32. Niijima T. Intravesical therapy and Adriamycin and new trends in the diagnostics and therapy of superficial urinary bladder tumours. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 37–44.
33. Edsmyr F, Berlin T, Boman J, et al. Intravesical therapy with Adriamycin in patients with superficial bladder tumours. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 45–53.
34. Fossberg E, and Sander S. Topical Adriamycin treatment of superficial bladder tumours. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 99–101.
35. Alfthan O, and Suominen R. Intravesical therapy with Adriamycin. *In* Diagnostics and Treatment of Superficial Urinary Bladder Tumors. Stockholm, Montedison Läkemedel AB, 1979, pp 95–98.
36. Duckek M. Local treatment of uroepithelial bladder tumours with Adriamycin. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
37. Jakse G, and Hofstadter F. Adriamycin therapy in carcinoma in situ: a preliminary report. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
38. Gammelgaard PA, Mogensen P, and Lundbeck F. Bladder instillation of Adriamycin in multiple recurrent non-invasive papillomatous bladder tumours. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
39. Collste L, Berlin T, Granberg-Öhman I, et al. Long term intracavitary Epodyl® in multiple or extensive papillary tumours of the urinary bladder. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
40. Ek A, and Collen S. Treatment of multiple non-invasive bladder tumours with intravesical Epodyl®. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.

41. Smith, JM, Lane V, and O'Flynn JD. Epdoyl® in management of noninvasive vesical neoplasms. Urology 11(5):474–477, 1978.
42. England HR, Blandy JP, and Paris AMI. The treatment of single and multiple papillary tumours of the bladder (T1/T1(a) NX M0). *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
43. National Bladder Cancer Collaboration Group A. The role of intravesical thiotepa in the management of superficial bladder cancer. Cancer Res 37:2916–2917, 1977.
44. Edsmyr F, and Andersson L. Chemotherapy in bladder carcinoma. Urol Res 6:263–264, 1978.
45. Esquivel EL (jr), Mackenzie AR, and Whitmore WF (jr). Treatment of bladder tumors by instillation of thiotepa, actinomycin D, or 5-fluorouracil. Invest Urol 2:381–386, 1965.
46. Jones HC, and Swinney J. Thiotepa in the treatment of tumours of the bladder. Lancet 2:615–618, 1961.
47. Pavone-Macaluso M. Chemotherapy of bladder tumours. Urol Res 6:259–262, 1978.
48. Smith PH. An approach to cytotoxic chemotherapy in carcinoma of the bladder. Proc Roy Soc Med. In press.
49. Yagoda A. Phase II trials of single agents and combination regimens in the treatment of urothelial tract tumours: Memorial Hospital experience. *In* Bladder Cancer–Principles of Combination Therapy (Hendry WF, Oliver RTD, and Bloom HJG, eds). London, Butterworth & Co, 1979. In press.
50. Turner AG, Durrant KR, and Malpas JS. A trial of bleomycin versus Adriamycin in advanced carcinoma of the bladder. Br J Urol 51:121-124, 1979.
51. Soloway MS. Cis-diamminedichloroplatinum II in advanced urothelial cancer. J Urol 120:716–719, 1978.
52. Oliver RTD. The place of chemotherapy in the treatment of patients with invasive carcinoma of the bladder. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
53. Smith PH, Akdas A, EORTC Urological Group, et al. Adriamycin and 5FU in the management of invasive bladder cancer. *In* Proceedings of the First Conference on the Treatment of Urinary Tract Tumours with Adriamycin, Tokyo, May 12, 1979. In press.
54. Lindholm CE, Mattsson W, Langeland P, et al. 5-fluorouracil and Adriamycin in locally recurrent and/or metastatic bladder cancer. *In* Bladder Tumours and Other Topics in Urological Oncology (Pavone-Macaluso M, Smith PH, and Edsmyr F, eds). London and New York, Plenum Press, 1979. In press.
55. Troner MD. Cyclophosphamide, Adriamycin and platinum (CAP) in the treatment of urothelial malignancy (Abstr) Proc Am Assoc Cancer Res 20:117, 1979.
56. EORTC Urological Group B. The treatment of advanced carcinoma of the bladder with a combination of Adriamycin and 5-fluorouracil. Eur Urol 3:276–278, 1977.
57. Sternberg JJ, Bracken RB, Handel PB, et al. Combination therapy (CISCA) for advanced urinary tract carcinoma. JAMA 238:2282–2287, 1977.
58. Williams SD, Einhorn LH, and Donohue JP. Cis-platinum combination treatment for bladder cancer. *In* Bladder Cancer–Principles of Combination Therapy (Hendry WF, Oliver RTD, and Bloom HJG, eds). London, Butterworth & Co., 1979. In press.
59. Martino S, Samal B, and Al-Sarraf M. 5-fluorouracil and Adriamycin in transitional cell carcinoma of the urinary tract: phase II. Personal communication. In press.
60. Al-Sarraf M, Vaughn CB, Reed M, et al. Combined 5-fluorouracil and vinblastine therapy for gastrointestinal and other solid tumours. Oncology 26:99–113, 1972.
61. Collier D, and Soloway MS. Doxorubicin hydrochloride, cyclophosphamide and 5-fluorouracil combination in advanced prostate and transitional cell carcinoma. Urology 8:459–464, 1976.
62. Rodriguez LH, Johnson DE, Holoye PY, et al. Combination V.M.-26 and Adriamycin for metastatic transitional cell carcinoma. Cancer Treat Rep 61:87–88, 1977.
63. Merrin C, Cartagena R, Wajsman Z, et al. Chemotherapy of bladder carcinoma with cyclophosphamide and Adriamycin. J Urol 114:884–887, 1975.
64. Pannuti F, Martoni A, Casadio M, et al. New regime of combination chemotherapy (CHVV) in the treatment of advanced bladder cancer: a pilot study. Cancer Treat Rep 63:1427–1428, 1979.
65. Turner AG, Hendry WF, Williams GB, et al. The treatment of advanced bladder cancer with methotrexate. Br J Urol 49:673–678, 1977.

Oral Methotrexate Therapy for Multiple Superficial Bladder Carcinomata [1]

R. R. Hall,* D. W. Herring,[2] A. C. McGill,[3] and I. Gibb[4]

SUMMARY

Patients with frequently recurring multiple class T1 bladder carcinomata have been treated with methotrexate (MTX) given as 50 mg in syrup every week. In 16 of 17 patients the frequency and number of tumor recurrences have been reduced. Measurement of MTX levels in the urine confirms that this dose of drug yields concentrations that are thought to be cytotoxic for at least 24 hours.

[Cancer Treat Rep 65 (Suppl. 1): 175-178, 1981]

The treatment of multiple superficial tumors of the bladder continues to be a problem despite advances in urological endoscopy and the use of a growing number of drugs instilled directly into the bladder. Evidence that cytotoxic drugs affect bladder carcinoma by direct transfer from the bladder lumen is scant, although in clinical practice it appears that some agents are moderately effective when applied in this manner.

In the hope of improving upon current methods of treatment we have used methotrexate (MTX) given systemically on the grounds that it could gain access to superficial tumors within the bladder both from the bloodstream and from the lumen of the bladder, since this drug is excreted mainly unchanged in the urine.

According to the current understanding of the absorption, metabolism, and excretion of MTX (1), and given the normal range of daily urine production, it should be possible to obtain cytotoxic concentrations of MTX in plasma and urine by the oral administration of 50 mg MTX. Furthermore, given normal renal function, this dose should be excreted safely within 36 hours so that the dose could be repeated weekly without toxicity. By this means, bladder tumors could be exposed to effective concentrations of drug in the plasma and urine more frequently and for longer periods of time than is achieved by catheterization and intravesical instillation of the drug.

PATIENTS AND METHOD

Patients presenting with very extensive multiple class T1 transitional cell carcinomata (2), or who had multiple T1 tumors at each cystoscopy for 1 year or more, were included in the study. Following initial cystoscopic assessment and after confirmation of normal renal function, liver function, and peripheral blood count, patients were treated with MTX syrup 50 mg orally once every week for a minimum of 6 weeks, after which they were recystoscoped to assess the extent of tumor necrosis.

Following this and regardless of the response to MTX at first followup cystoscopy, the bladder of each patient was rendered tumor free by endoscopic resection, the latter being repeated after a few weeks if necessary to ensure complete tumor clearance. Thereafter the patients were maintained on the same dose of oral MTX for 18 months to assess its effectiveness in the prevention of tumor recurrence, as assessed by regular cystoscopy at approximately 3-month intervals. All tumors, regardless of size, were included and counted as separate lesions in calculating tumor incidence and recurrence. Complete blood counts were performed each week before administration of MTX, and the dose was withheld if total wbc count was less than 3,000 or the total platelet count was less than 100,000. At the time of each cystoscopy, renal function and liver function were assessed and chest X-ray performed.

METHOTREXATE ASSAY

At the commencement of therapy and at the time of each cystoscopy all urine passed by the patient was

[1] Methotrexate assay facilities have been made available by a donation from the Chevington Comrades Club, Red Row, Northumberland.

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collected by an indwelling catheter in 4-hour aliquots for 24 to 48 hours following administration of MTX. Urinary MTX concentration was measured by means of radioimmunoassay developed in our own laboratory employing ^{125}I-MTX. This assay employs a high avidity antiserum that allows use of a short incubation period at an elevated temperature and a rapid charcoal separation stage. This, in combination with the use of a multicrystal gammacounter, enables rapid availability of results. The sensitivity of the assay was 0.2 μg/l and the interassay precision was 5% to 10% at the urinary levels of MTX found in this study. There was no significant cross reactivity with the major endogenous folates.

Previously published (3) urinary concentration data from some patients in this study were obtained using the radioimmunoassay described by Aherne et al (4), which employed tritiated MTX. The limiting factors of this assay were the poor throughput time and the other inherent disadvantages of a liquid scintillation technique. For these reasons, we developed the assay outlined above.

RESULTS

Primary Treatment

Fourteen patients received oral MTX as primary therapy. In no patient has total tumor necrosis occurred. In 5 of 14 patients the number and size of tumors were reduced by more than 50% when assessed by cystoscopy after 6 to 16 weeks of MTX therapy. In the remaining nine patients tumor remained unchanged or was more extensive.

Maintenance Therapy

Seventeen patients are currently receiving maintenance MTX therapy. Average followup to date is 8.9 months (range 2 to 19 months). Seven patients were tumor free at their last cystoscopy and in nine others the frequency and number of tumor recurrences have been reduced. MTX therapy has failed in one, the disease progressing rapidly and cystectomy being necessary within 3 months.

In the 12-month period prior to starting MTX treatment, 17 patients underwent 63 cystoscopies, of which 61 revealed tumor present and only 2 were tumor free. Following commencement of MTX therapy, 36 cystoscopies were performed, of which 21 revealed recurrent tumor and 15 were tumor free.

Prior to MTX therapy the overall incidence of tumors was 9.5/cystoscopy (9.8/cystoscopy when tumor present). Following MTX, tumor incidence was only 2.1/cystoscopy overall (4.1 tumors/cystoscopy, tumor cases only). With the exception of the patient who proceeded rapidly to cystectomy, no patient has had more than eight recurrent tumors at last cystoscopy, and the majority of these recurrent tumors have been less than 5 mm in diameter.

Toxicity

Only one incident of serious toxicity occurred, in a patient who misunderstood the instructions for medication and took 50 mg MTX daily for 1 week. He developed severe oral and skin ulceration, moderate diarrhea, leucopenia, and thrombocytopenia. All symptoms and signs subsided following 10 days of oral leucovorin therapy. This patient subsequently received 50 mg MTX weekly without further toxicity.

Two other patients complained of mucositis and oral ulceration following their first or second dose of MTX, but this did not occur with subsequent doses. Two patients complained of burning discomfort in the eyes, which was relieved by leucovorin eye drops, but did not interfere with therapy. A total of 428 doses of weekly oral MTX were administered, and on 3 occasions only was therapy deferred because of thrombocytopenia. Significant leucopenia was not observed.

URINE METHOTREXATE LEVELS

Urinary MTX concentrations were measured on 16 separate occasions in 12 patients. The results (fig 1) show a wide variation in MTX concentration; but it should be noted that on each occasion the urinary concentration exceeded 10^{-6} M for 24 hours following oral administration. Drug concentration at 4 hours was approximately 5×10^{-5} M and at 8 hours was 3×10^{-5} M.

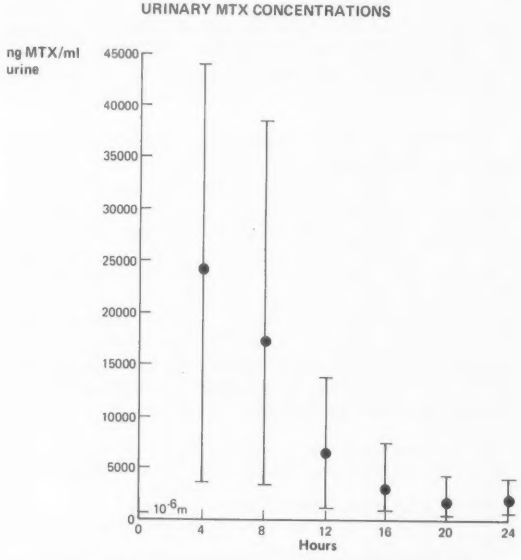

FIGURE 1.—MTX concentration in urine of patients receiving 50 mg oral MTX. Results shown as mean value and range.

The average retrieval of MTX in the urine within 24 hours was 41% of the oral dose (range 10% to 79%). Variation within individual patients has also been considerable.

Figure 2 shows the pattern of MTX excretion: the majority of drug excreted in the urine appeared within the first 8 hours.

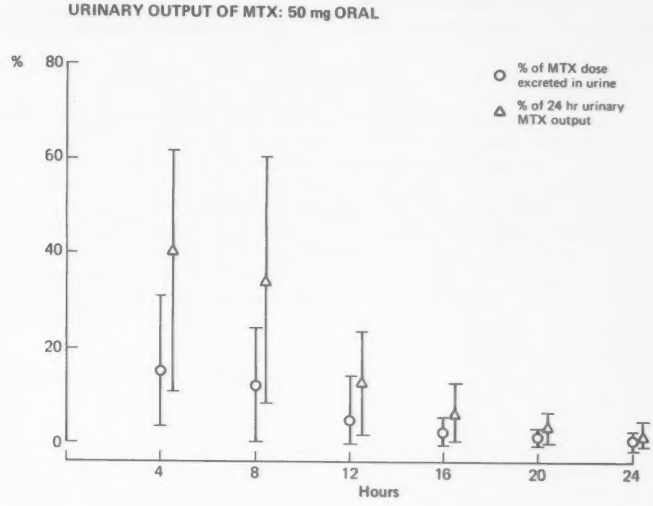

FIGURE 2.—Pattern of MTX excretion in urine following 50 mg oral dose MTX. Results shown as mean plus range.

DISCUSSION

At the present time there is no universal agreement on the definition of the term "multiple superficial tumors of the bladder." To some, "multiple" means more than 2, while others will use the term only when describing a bladder completely smothered with 40 or 50 separate tumors or a bladder in which the majority of the surface is covered by confluent tumor. The size of papillary bladder tumors is also difficult to document. A 2 cm papillary tumor is larger than a 1 mm lesion, obviously, and is described by some urologists as a large tumor. To another urologist a 2 cm lesion may be resected endoscopically with the greatest of ease, and that urologist would consider the tumor large only if greater than 5 cm in diameter.

These distinctions may appear rather mundane, but they are of particular importance at the present time because of the widespread current interest in the management of multiple superficial tumors of the bladder, and the number of trials of various cytotoxic agents currently in progress. For example, when assessing the results of treatment, shall we attach the same significance to the disappearance or recurrence of ten 2 mm tumors in the bladder as that for a single 2 cm tumor? The cystoscopic documentation of the extent of this disease is subjective, to say the least, but there is a need for agreement on the minimum size and number of tumors that shall be accepted for inclusion in clinical studies. For the purpose of this report, it should be understood that only those patients whose multiple, frequently recurring tumors had become a problem for endoscopic management were included in the study. Furthermore, in calculating tumor incidence and recurrence, all tumors of whatever size were included and counted as separate lesions.

The levels of MTX measured in the urine to date are encouraging, for they verify in practice one of the principles of this particular study, namely that an oral dose of 50 mg MTX yields concentrations that are believed to be cytotoxic for a longer period than may be achieved by conventional intravesical instillation. The absence of serious toxicity endorses the safety of this regimen and suggests that it could be used in a much larger patient population without undue risk.

In a previous study of a similar group of patients with multiple superficial bladder carcinomata, 80% of patients developed recurrences within 1 year of treatment, the majority of whom required subsequent

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radical therapy.[5] Compared with this, the results of oral MTX therapy to date appear encouraging.

So far only one patient has completed 18 months of therapy, and thus no data are available on the behavior of these patients' bladders after withdrawal of therapy. Further followup is necessary before statistical analysis will be meaningful. From the clinical point of view our results are regarded as favorable, for in 15 of 17 patients their bladder disease became much more easily manageable since commencing maintenance MTX therapy.

[5] Hall, unpublished.

REFERENCES

1. Bleyer WA. Methotrexate: clinical pharmacology, current status and therapeutic guide lines. Cancer Treat Rev 4:87–101, 1977.
2. Union Internationale Contre le Cancer. Classification of malignant tumors, 3d ed. Geneva, 1978.
3. Hall RR. Methotrexate therapy for multiple stage T1 bladder carcinoma. *In* Bladder Tumors and Other Topics in Urological Oncology, Ettore Majorana International–Life Sciences Series, vol 1. New York, NY, Plenum Press. In press.
4. Aherne GW, Piall EM, and Marks V. Development and application of a radioimmunoassay for methotrexate. Br J Cancer 36:608, 1977.

Methotrexate as Salvage or Adjunctive Therapy for Primary Invasive Carcinoma of the Bladder

R. T. D. Oliver*

SUMMARY

A phase II study of methotrexate (MTX) treatment of recurrent primary invasive tumors of the bladder is reported. The incidence of measurable response is lower than that seen when the drug is used to treat patients with metastatic disease. However, the overall response achieved when durable symptomatic responses are included, and the absence of major side effects, establishes this drug as a good palliative treatment. Results from a pilot study giving MTX prior to radiotherapy demonstrate that it is possible to combine the two treatments without increasing toxicity. The early results from this study were better than a previously treated historical control series and justify evaluation of adjuvant chemotherapy with MTX in a prospectively randomized trial.

[Cancer Treat Rep 65 (Supp. 1): 179–181, 1981]

In patients treated by radical radiotherapy, failure of local control is the principal cause of death in approximately two-thirds of the patients who die (1) (table 1). After preoperative radiotherapy and cystectomy, local recurrence as principal cause of death occurs in about 30% of patients (2). Though this difference justifies the use of cystectomy, this combined approach does not lead to a significant increase in long-term survivors (3), particularly in patients over the age of 65 (ie, more than 50% of patients). This emphasizes the need for new approaches to the management of the primary tumor.

TABLE 1.—Survival of patients following radical radiotherapy

	Disease status at last observation			
	T1	T2	T3	T4
	n=90	n=132	n=220	n=262
Alive	48%	23%	29%	4%
(% disease free)	(71)	(87)	(79)	(55)
Dead, local disease	21%	25%	23%	40%
Dead, local disease + Mets	10%	18%	15%	31%
Dead, metastasis	2%	5%	5%	8%
Dead, other	16%	25%	23%	14%

Followup from 5 to 15 years. (Data from Hope-Stone et al [1].)

Most of the information on chemotherapy in carcinoma of the bladder has been obtained from studies on patients with measurable metastatic disease. However, from the earliest days of intra-arterial infusion, most authors have reported some response of primary tumors. As has been shown by Turner et al (4), methotrexate (MTX) is an active drug as salvage treatment in patients with metastatic bladder cancer, but information on its activity against recurrent primary tumors is limited. This paper reviews the experience at the Institute of Urology using MTX 100 mg/m² followed by folinic acid (FA), given weekly as salvage treatment for patients whose primary tumor has recurred after radiotherapy, and reports on a pilot study evaluating the combination of MTX and radiotherapy as primary treatment in previously untreated patients.

MTX AS SALVAGE THERAPY FOR RECURRENT PRIMARY TUMORS OF THE BLADDER

Patients and Methods

Twenty-eight patients with locally recurrent bladder cancer received salvage treatment with weekly MTX 100 mg/m² followed by FA. In patients with a pretreatment creatinine clearance >80 ml/minute, one im dose of FA 15 mg was given 24 hours after the MTX bolus. In patients with a creatinine clearance between 50 to 80 ml/minute, 15 mg oral FA were given every 6 hours for eight doses. One patient who had a cystectomy and two patients who had previously had a nephrectomy presented with metastases

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only. Of the 31 patients, 9 had clinically measurable metastases and 16 had no detectable metastases.

RESULTS AND DISCUSSION

Response rates to MTX are summarized in table 2. Of the nine patients with metastases, three (33%) had a response in metastatic sites. There were no measurable responses in bone metastases, although two out of six patients had significant reduction in bone pain. Of 16 patients whose only site of disease was the bladder, 4 (25%) had a response. This figure is somewhat higher than previously reported (4,5), although this discrepancy may be explained by the fact that the series differed in terms of the T stage of tumors. Response rate in this series appeared to be influenced by the size of the primary tumor; the smaller the primary tumor the higher the response rate (table 3).

TABLE 2.—MTX as salvage treatment for recurrent transitional cell carcinoma

	Number of patients	CR + PR	MR
Measurable metastases	9	33%	0
Bone metastases	6	0	33%
Primary tumor	16	25%	38%

CR=Complete response.
PR=> 50% reduction measurable mass.
MR=Either minimal change in measurable lesion or significant symptomatic improvement for > 6 weeks.

TABLE 3.—MTX as salvage treatment for recurrent transitional cell carcinoma

[Influence of patient TNM stage on response]

	N	CR	PR	MR
≤ T3M0	6	50%	0	0
≤ T3M+ (and/or N+)	8	13%	26%	13%
T4 M0	10	0	10%	60%
T4 M+ (and/or N+)	7	0	0	14%

MTX AS ADJUNCT TO RADIOTHERAPY IN PREVIOUSLY UNTREATED PATIENTS

On the basis of these results, which confirmed previous reports (4,5), ie, that MTX is an active single agent against bladder cancer, a pilot study was commenced to assess the value of MTX when added to radiotherapy in the primary treatment of invasive bladder cancer.

Nineteen patients with T2 to T4 tumors were given three courses of MTX 100 mg/m² with FA rescue at weekly intervals prior to starting radiotherapy, and thereafter at 2-week intervals for 1 year. Table 4 shows the disease status of the patients at 6 months, and table 5 shows the survival of the patients at 12 months. The numbers of patients treated are too small to draw conclusions, and further patient accrual will be necessary before this combined treatment regimen can be compared to the results reported for radiotherapy alone (1). MTX was well tolerated in this pilot study, with the only toxicity being oral ulceration in two patients.

TABLE 4.—MTX as an adjunct to RXT in the treatment of primary bladder tumors

	Influence on CR rate at 6 months		
	T2	T3	T4
RXT alone * (number of patients)	48%	42%	6%
Historical controls	(121)	(196)	(224)
MTX	75%	62%	11%
+ RXT	(4)	(13)	(9)

* Data from Hope-Stone et al (1).

TABLE 5.—MTX as an adjunct to RXT in the treatment of primary bladder tumors

	Influence on CR 12-month survival		
	T2	T3	T4
RXT alone	58%	58%	38%
(Historical controls) *	(121)	(196)	(224)
MTX	67%	67%	43%
+ RXT	(3)	(9)	(7)

* Data from Hope-Stone et al (1).

CONCLUSIONS

Results from the study of salvage MTX in patients with recurrent bladder cancer confirm the activity of the drug against this tumor. In our hands, the response rate to MTX is significantly higher than that obtained previously with Adriamycin or bleomycin, and is slightly higher and more durable than that obtained with cis-platinum. Furthermore, there were negligible complications due to MTX. The results of the pilot study of adjunctive MTX in the primary treatment of bladder cancer are encouraging, but a prospective randomized trial is required to adequately define its long-term value.

REFERENCES

1. HOPE-STONE HF, OLIVER RTDO, ENGLAND HR, ET AL. Combination treatment for carcinoma of the bladder. London, Butterworth, 1980. In press.

2. Whitmore WF, Batata MA, Ghoneim MA, et al. Radical cystectomy with or without prior irradiation in the treatment of bladder cancer. J. Urol 118:184–187, 1977.
3. Wallace DM, and Bloom HJG. The management of deeply infiltrating (T3) bladder carcinoma: controlled trial of radical radiotherapy versus pre-operative radiotherapy and radical cystectomy (First Report). Br J Urol 48:587–594, 1976.
4. Turner AG, Hendry WF, Williams GB, et al. The treatment of advanced bladder cancer with methotrexate. Br J Urol 49:673–678, 1977.
5. Hall RR, Bloom HJG, Freeman JE, et al. Methotrexate treatment for advanced bladder cancer. Br J Urol 46:431–438, 1974.

Methotrexate in Advanced Bladder Cancer

A. G. Turner*

SUMMARY

Over a period of 9 years (1970 to 1979), 64 patients with advanced local or metastatic bladder cancer were treated with methotrexate by one of three regimens. Response rates of 50% and over were noted with two of the three regimens studied. The response rate was greater for metastatic disease (44%) than for local disease (16%). Toxicity at the dosage used was minimal provided renal function was satisfactory.

[Cancer Treat Rep 65 (Suppl. 1): 183–186, 1981]

Sullivan (1) gave methotrexate (MTX) intra-aortically to patients with advanced bladder cancer and noted a sustained clinical benefit and decrease in tumor size. Further reports (2–5) supported this finding, but each report contained only a few cases. There was, however, an overall response rate of 36%. Hall (6) performed a much larger study, in which 42 patients with either local or metastatic disease were treated with MTX, and a 26% response rate was recorded. The latter study has been extended and reported (7). This report concerns a further extension of this study and updates the results.

PATIENTS

Patients with advanced local disease that was refractory to radiotherapy or had recurred after radiotherapy and patients with metastatic disease were considered for therapy. Information on patient performance status is not available, as this is a retrospective review.

Pretreatment investigations included hgb, wbc, platelets, urea, creatinine, glomerular filtration rate (GFR), either creatinine clearance or EDTA clearance, chest X-ray, and bone scan or skeletal survey. If the wbc count was found to be below 3×10^9/liter, and/or the platelet count was below $100{,}000 \times 10^9$/liter, then the treatment was not given. Counts were checked 2 weeks later and, if satisfactory, treatment was resumed.

Toxic side effects were noted, and if mild, treatment was temporarily stopped, but if severe, treatment was abandoned.

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TREATMENT REGIMENS

Three different regimens were used, each given at 2-week intervals:

A. MTX 50 mg iv. *No* folinic acid (FA) rescue.
B. MTX 100 mg iv. *No* FA rescue.
C. MTX 200 mg im with FA rescue (21 mg im) at 6, 12, and 24 hours.

Regimens A and B without FA rescue were given on an outpatient basis. Regimen C was given on an inpatient basis because of the requirement for im FA rescue (the oral form was not available when this study was instituted).

RESULTS

During the period 1970 to 1979, 64 patients were treated with one of the regimens outlined above: 25 patients were given regimen A, receiving between 1 and 47 courses with an average number of 10; 25 patients were given regimen B, receiving between 2 and 64 courses with an average of 17.5; 16 patients were given regimen C, receiving between 3 and 49 courses with an average of 13.0. No patients died of MTX toxicity; all deaths were due to disease progression.

Both objective and subjective responses were noted, but only objective responses are considered in this analysis. Objective responses are defined as: complete response (CR)—a complete regression of tumor lasting at least 6 months; partial response (PR)—50% reduction in the sum of two parameters in two dimensions of measurable metastases and/or downstaging of local bladder tumors, the latter being assessed

under general anesthesia and confirmed by more than one observer on more than one occasion.

Complete Responses

Four CR's were noted. Sites and duration of these responses are described in table 1. As can be seen, there were no CR's in the 50 mg dose group, three in the 100 mg group, and one in the 200 mg with rescue group. The patient who had a CR in bone had been admitted with paralysis due to spinal metastases. He is now able to walk and has returned to work. The patient with pulmonary metastases who received 200 mg with rescue and achieved a CR died 12 months after disappearance of the lung lesion (fig 1, 2). As the lung lesion cleared and MTX was discontinued, a solitary spinal metastasis developed and the patient died. At post mortem the lung was found to be clear.

TABLE 1.—Complete objective response: duration of remission for specific metastatic sites

Complete objective response	Duration of remission in months (MTX every 2 weeks)		
	50 mg	100 mg	200 mg with rescue
Local	-	-	-
Lung	-	19	22
Bone	-	18	-
Skin	-	7	-
Total numbers	0/25	3/23	1/16

Partial Responses

There were nineteen PR's. The sites and durations of the responses are described in table 2. One patient with a local response of 87 months' duration on the 50 mg regimen is still alive, MTX treatment having been stopped after 47 courses. He was originally staged as T3, but after radiotherapy was staged T4 and totally inoperable at laparotomy. Tumor fixation remains reduced and there is no evidence of metastatic disease.

TABLE 2.—Partial objective response: duration of remission for specific metastatic sites

Partial objective response	Duration of remission in months (MTX every 2 weeks)		
	50 mg	100 mg	200 mg with rescue
Local	89	4, 7, 26	4, 6, 34
Nodal*	-	3	4
Lung	6	8, 8, 12	4, 3, 4
Bone	-	2, 4, 14	3, 3
Skin	2	3	-
Total numbers	3/25	9/23	7/16

*Nodal response was defined by lympography, there being a >50% reduction in radiographic size of lymph node.

To summarize these results, ie, CR × PR, there were few responses (12%) in the 50 mg group, a large number (52%) in the 100 mg group, and 50% in the 200 mg with rescue group.

Survival

The survival rates from onset of treatment with MTX to death are shown in table 3. The average survival of the nonresponder is between 9.5 and 6.75 months in each group. Patients who responded to treatment showed an increase in survival. Comparison can be drawn only between the nonresponders and partial responders in groups B and C where the numbers are sufficient. In these groups the average survival of responders was 16.5 and 12.0 months compared with 7.0 and 6.75 months for the nonresponders. These results suggest that MTX is an effective agent against bladder cancer.

TABLE 3.—Survival by response and regimen

Response	Survival in months		
	50 mg	100 mg	200 mg with rescue
None	1 to 48 av 9.5	1 to 12 av 7.0	2 to 12 av 6.75
Partial	10 to 89 av 38	3 to 60 av 16.5	2 to 36 av 12
Complete	–	7 to 20 av 15	22

Subjective Responses

Five patients in the study group experienced dramatic pain relief after administration of MTX. In each case the pain was from bony metastases. Despite marked pain relief, there was no objective evidence of regression.

Toxicity

Toxicity was classified as mild or severe; in mild cases therapy was temporarily stopped, but in severe cases treatment was abandoned. The most commonly observed side effect was mucositis; 19 patients (29%) suffered from mucositis, but in only two cases was the condition so severe that treatment had to be abandoned. In total only five patients had such severe side effects that treatment had to be abandoned. In addition to the two patients with severe mucositis, one suffered from skin necrosis while receiving concurrent D & T, one had severe marrow toxicity, and one had a lung perfusion defect.

DISCUSSION

Since the review of chemotherapy in urological malignancy by Carter and Wasserman (8), there have been numerous reports and trials in an attempt to find new agents or combinations of agents for the treatment of bladder cancer. Agents shown to be of value from these studies were Adriamycin (8) and cisplatinum (9). This study shows that MTX is an effective chemotherapeutic agent in bladder cancer, the optimum regimens in this study having produced a response rate of about 50%.

Effects on local and metastatic disease should be considered separately as there is a critical need for improvement in the control of local disease (10,11).

In this study, only 7 of 39 patients (16%) with local stage T4 tumor showed any response. This finding is in accordance with previous reports (2,5,6) and may be due to the effects of previous radiotherapy, response to the drug (12), or the advanced stage of the tumor, which may render it resistant to all forms of treatment (13).

A 44% response rate, which was found in metastatic disease, was also noted by Hall et al (6).

Since MTX is excreted by the kidneys, pretreatment renal function assessment is essential. Patients with advanced bladder cancer may have impaired renal function (because of the disease and advanced age), and MTX itself can also cause renal tubular damage (14).

In this study, MTX was well tolerated and there was no evidence of renal tubular damage. Few of the unpleasant and toxic side effects associated with other agents (9,15,16) were found. The higher incidence of toxicity seen in the 50 mg group was due to inexperience in using the drug and initial attempts to use it on a weekly basis. It soon became obvious that the practical dose regimen was every 2 weeks. Severe side effects were uncommon.

CONCLUSION

From this study, it is concluded that MTX is a valuable chemotherapeutic agent in the management of bladder cancer. It is a safe, well-tolerated drug with few side effects and can be used on an outpatient basis. Its greatest effect is against metastatic disease, and therefore it should be evaluated in combination with established treatments in the primary therapy of invasive bladder cancer.

REFERENCES

1. Sullivan RD. Intra-arterial methotrexate therapy: the dose duration and route of administration studies of methotrexate in clinical cancer chemotherapy. *In* First Symposium on Methotrexate in the Treatment of Cancer, (Porter R, and Wiltshaw E, eds). Bristol, Wright, 1962, pp 50–55.
2. Burn JI. Intra-arterial infusion in malignant diseases of the pelvis. *In* Second Symposium on Methotrexate in the Treatment of Cancer, (Worrall PM, and Espiner HJ, eds). Bristol, Wright, 1966, pp 58–63.
3. Andrews NC, and Wilson WL. Phase 2 study of methotrexate in solid tumours. Cancer Chemother Rep 51:471–474, 1967.
4. Pavone-Macaluso M. Chemotherapy of vesical and prostatic tumours. Br J Urol 43:701–708, 1971.
5. Altman CC, McCague NJ, Ripedi AC, et al. The use of methotrexate in advanced carcinoma of the bladder. J Urol 108:271–273, 1972.
6. Hall RR, Bloom HJG, Freeman JR, et al. Methotrexate treatment for advanced bladder cancer. Br J Urol 46:431–438, 1974.
7. Turner AG, Hendry WF, Williams GB, et al. The treatment of advanced bladder cancer with methotrexate. Br J Urol 49:673–678, 1977.
8. Carter SK, and Wassorman TH. The chemotherapy of urological cancer. Cancer 36:729–747, 1975.
9. Yagoda A, Watson RC, Gonzalez-Vitale JC, et al. Cis-dichlorodiammineplatinum (ii) in advanced bladder cancer. Cancer Treat Rep 60:917–923, 1976.
10. Miller A, Mitchell JP, and Brown NJ. The Bristol Bladder Tumour Registry. Br J Urol 41 (Suppl):17–43, 1969.
11. Wallace DM and Bloom HJG. The management of deeply infiltrating (T3) bladder cancer—controlled trial of radical radiotherapy versus preoperative radiotherapy and cystectomy. (First report). Br J Urol 48:587–594, 1976.
12. Johnston IDA. An assessment of intra-arterial infusion with methotrexate in the treatment of malignant disease of the head and neck. *In* Second Symposium of Methotrexate in the Treatment of Cancer, (Worrall PM, and Espiner JH, eds). Bristol, Wright, 1966, pp 52–57.
13. Irvine WT. *In* Second Symposium of Methotrexate in the Treatment of Cancer, (Worrall PM, and Espiner JH, eds). Bristol, Wright, 1966, p 68.
14. Condit PT, Chanes RE, and Joel W. Renal toxicity of methotrexate. Cancer 23:126–131, 1969.
15. Turner AG, Durrant K, and Malpas JS. A trial of bleomycin versus Adriamycin in advanced carcinoma of the bladder. Br J Urol 51:121–124, 1979.
16. Glashan RW, Houghton AL, and Robinson MRG. A toxicity study of the treatment of T3 bladder tumours with a combination of radiotherapy and chemotherapy. Br J Urol 49:669–672, 1977.

Surgery and Adjuvant Chemotherapy with High-Dose Methotrexate and Folinic Acid Rescue for Infiltrating Tumors of the Bladder

Y. Socquet*

SUMMARY

Thirty-three patients with category T3 or T4 bladder cancer were treated by partial or total cystectomy followed by 6 to 12 courses of high-dose methotrexate (HDMTX) with folinic acid rescue. Survival at 2 years was 90%. Two patients were treated palliatively with HDMTX alone, one of whom is alive and disease free after 35 months.

[Cancer Treat Rep 65 (Suppl. 1): 187–189, 189]

Studies of the activity of single agents against advanced bladder cancer have identified three active drugs, namely Adriamycin (1), cis-platinum (2), and methotrexate (MTX) (3–6); the latter two are apparently comparable in activity. Clinical pharmacology studies with MTX (7) have shown that, provided certain precautions are observed and patients satisfy various pretreatment requirements, MTX is well tolerated and has a low incidence of the toxic side effects associated with other cytotoxic drugs. MTX was therefore chosen as adjuvant chemotherapy to surgical treatment by partial or total cystectomy of category T3 or T4 bladder cancer in the hope of improving upon the unsatisfactory results obtained from surgery or radiotherapy alone or in combination. High-dose MTX (HDMTX) was used in an attempt to overcome natural or acquired resistance to the drug.

PATIENTS AND METHODS

Patients were staged according to the classification of the Union Internationale Contre le Cancer (UICC Geneva 1978). The N category was based on clinical examination, radiography, lymphography, urography, and CT scan. Metastatic status was assessed by clinical examination, radiography, and bone scan. Liver scans were performed if clinically indicated.

PRETREATMENT INVESTIGATIONS

Prior to and 12 days after administration of HDMTX, total wbc and platelet counts were determined. Minimum pretreatment leucocyte and platelet levels were 3,000/m³ and 100,000/m³ respectively. Serum creatinine determinations were made immediately prior to treatment and 24 hours later. Minimum pretreatment creatinine levels were 1.2 mg% in females and 1.5 mg% in males. If there was a rise of 50% or more in the serum creatinine at 24 hours the dose and duration of folinic acid rescue were increased.

ADMINISTRATION OF HDMTX

MTX (1 to 2 g) was given by iv infusion in 1 liter normal saline over 6 hours. Six hours after the end of the MTX infusion of folinic acid, 9 to 12 mg were given iv, im, or orally every 6 hours for eight doses. Urinary pH was made alkaline prior to the MTX infusion by administration of oral sodium bicarbonate. Patients were instructed to drink a minimum of 3 liters fluid/24 hours starting 24 hours before the MTX infusion and continuing for 3 days to maintain a urinary output >2.5 liters/24 hours. Patients gargled every 3 hours for 2 days from the end of the infusion with the following solution:

Polymyxin	2.25 mg
Neomycin	166.25 mg
Bacitracin	215.00 mg

Aurantii Corticus Syrup to 1 liter.

In addition, patients were instructed to swallow 3.5 ml Nystatin drops (100,000 iu/ml) or to chew a tablet of Nystatin (500,000 iu) every 3 hours. Nausea and vomiting were controlled by antiemetics and iv hydration as necessary. Chemotherapy was commenced 6 weeks after cystectomy and continued at 3-week intervals for a minimum of 6 months.

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RESULTS

Thirty-three patients with category T3 or T4 transitional cell lesions received adjuvant chemotherapy with HDMTX following partial or total cystectomy. A further two patients received HDMTX as palliative therapy only.

The TNM classification of the 35 patients before surgery and the postsurgery histopathological grading are shown in table 1.

TABLE 1.—Distribution of patients according to tumor stage and treatment

Group	Treatment	Number of patients	Histological grading
A T3aN0M0	Partial cystectomy followed by HDMTX—6 courses.	20	G1 = 6 G2 = 10 G3 = 4
B T3aN+M0	Partial cystectomy + regional lymphadenectomy followed by HDMTX—9 courses.	5	G2 = 2 G3 = 3
C T3bN0M0	Total cystectomy followed by HDMTX—9 courses.	3	G3 = 3
D T3bN+N0	Total cystectomy followed by HDMTX—12 courses.	2	G2 = 1 G3 = 1
E T4aN0M0	Total cystectomy followed by HDMTX—12 courses.	3	G3 = 3
F	HDMTX palliative 1 patient: T3bN0M0 received HDMTX for 24 months; 1 patient: T4N4M1 died after 11 months.		G3 = 2
Total	33 patients: cystectomy + HDMTX; 2 patients: HDMTX.		35

NB: HDMTX was initially 1 g and all subsequent courses 2 g.

Table 2 shows the actual survival of the patients with category T3 or T4 lesions treated by partial or total cystectomy followed by HDMTX. Thirty-one of the thirty-three patients have a minimum followup of 12 months and all were alive and disease free at this time. Of 20 patients with 24-month followup, 18 were alive and disease free. Of 10 patients with followup > 36 months, 6 were alive and disease free.

TABLE 2.—Survival of patients with T3 or T4 bladder tumors treated by partial or total cystectomy + HDMTX

Followup	Number of patients	Number surviving	Percent survival
>12 months	31	31	100
>24 months	20	18	90
>36 months	10	6	60

Of the two patients treated palliatively, one patient with T3bN0M0 disease is alive and disease free after 39 months. The other patient with T4N4M1 disease died of metastatic disease at 11 months. Causes of death are summarized in table 3.

Only one treatment complication was observed, namely an ulcerous stomatitis associated with bullous dermatitis in a patient with slight renal impairment (creatinine 2.20 mg%) following iv infusion of MTX 1 g.

DISCUSSION

These results using HDMTX as adjuvant therapy to partial or total cystectomy for patients with category T3 or T4 bladder tumors suggest significant activity

TABLE 3.—Causes of death

	Total number of patients	Deaths/complications	Number surviving
Group A = T3aN0M0	20	0	20
Group B = T3aN+M0	5	-17 months: death from generalized metastases (G3) -21 months: death from cerebral metastases (G3)	3
Group C = T3bN0M0	3	-33 months: deceased following myocardial infarction (G3)	2
Group D = T3bN+M0	2	0	2
Group E = T4aN0M0	3	-27 months: deceased following pathological fracture and embolism (G3)	2
Group F = Palliative	2	-11 months: deceased (G3)	1
Total	35		30

of MTX against bladder cancer. The absence of local recurrence in the bladder after partial cystectomy is particularly noteworthy.

While it is early to draw definitive conclusions from this study, these results are superior to those reported previously for surgery or radiotherapy alone (8–10). They are also superior to those results reviewed by Carter and Wasserman (11) for surgery combined with radiotherapy, and to recently reported results (12–14) in which various combinations of surgery and radiotherapy were employed.

CONCLUSION

HDMTX as adjuvant therapy to partial or total cystectomy for patients with category T3 and T4 lesions appears to be highly effective in preventing recurrence and prolonging survival. In addition, treatment was well tolerated with a low incidence of toxic side effects.

ADDENDUM

In the 6 months that have passed since making this preliminary analysis, there have been no further recurrences or deaths.

REFERENCES

1. O'Bryan RM. Phase II evaluation of Adriamycin in human neoplasia. Cancer 32:9–17, 1973.
2. Yagoda A. Cis-diamminedichloroplatinum in advanced bladder cancer. Cancer Treat Rep 60:917–923, 1976.
3. Burn JI, Johnson ID, Davis AJ, et al. Cancer chemotherapy by continuous intra-arterial infusion of methotrexate. Br J Surg 53:329–336, 1966.
4. Altman CC, McCague NJ, Ripepi AC, et al. The use of methotrexate in advanced carcinoma of the bladder. J Urol 108:271–273, 1972.
5. Hall RR, Blood HJG, Freeman JF, et al. Methotrexate treatment for advanced bladder cancer. Br J Urol 46:431–438, 1974.
6. Turner AG, Hendry WF, Williams GB, et al. The treatment of advanced bladder cancer with methotrexate. Br J Urol 46:673–678, 1977.
7. Isacoff WH, Townsend CM, Eilber FR, et al. High dose methotrexate therapy of solid tumors: observations relating to clinical toxicity. Med Paediatr Oncol 2:319–325, 1976.
8. Jewett JJ. Treatment of carcinoma of the bladder—a summary. *In* Proceedings of the Sixth National Cancer Conference. Philadelphia, PA, JB Lippincott, 1970, pp 11–217.
9. Cuccia CA. Cancer of the urogenital tract—bladder cancer—the radiation attack. JAMA 206:347–348, 1969.
10. Kuohara SS, Rubin P, and Solin N. Analysis in depth of bladder cancer treated by supervoltage therapy. Am J Roentgenol 95:458–467, 1965.
11. Carter SK, and Wasserman TH. The chemotherapy of urology cancer. Cancer 36:729–747, 1975.
12. Van der Werf, MB. Carcinoma of the bladder T_3N+M0 treated by pre-operative irradiation followed by cystectomy—third report of the Rotterdam Radiotherapy Institute. Cancer 36:718–722, 1975.
13. Miller LS. Bladder cancer—superiority of preoperative irradiation and cystectomy in clinical stages B2 and C. Cancer 39:973–980, 1977.
14. Ellingwood KE, Drylie DM, De Ture FA, et al. Post-diversion precystectomy irradiation for cancer of the bladder. Cancer 43:1032–1036, 1979.

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